

CLOWING OF THE ENOLASE GENE FROM
ESCHERICHIA COLI AND CHARACTERIZATION
OF A MULTIMERIC ENOLASE FROM
SULFOLOBUS ACIDOCALDARIUS

CENTRE FOR NEWFOUNDLAND STUDIES

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**Cloning of the Enolase Gene from
Escherichia Coli and
Characterization of a Multimeric Enolase
from Sulfolobus Acidocaldarius**

By



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A thesis submitted to the School of Graduate
Studies in partial fulfillment of the
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Abstract.

Glycolytic enzymes involved in three-carbon metabolism are found in representatives of every phylogenetic lineage. As these enzymes evolve slowly and their amino acid sequences are highly conserved, these proteins and their genes should be important tools for studying long term evolutionary relationships. Enolase was the glycolytic enzyme chosen for this thesis. A hybrid recombinant plasmid, pLC10-47, is known to complement an *Escherichia coli* enolase mutant (Thomson *et al.*, (1979), J. Bacteriol. 137: 502-506). As a start to comparing the structure of the enolase gene from *E. coli* with its eukaryotic counterparts, the location of the *E. coli* enolase gene on pLC10-47 was determined. This was achieved by a process of restriction endonuclease digestion of pLC10-47, subcloning the fragments into pUC 12 and transforming *E. coli* with recombinant plasmids. Plasmid DNA was isolated from the transformants and examined for the presence of DNA from pLC10-47. Cell extracts of the transformants were assayed for enolase activity. The *E. coli* enolase gene was localized to the region around the unique Eco RI site of pLC10-47. As part of a study designed to compare the enolases from representatives of the three primary kingdoms, the enolase from *Sulfolobus acidocaldarius* was partially purified and characterized. *S. acidocaldarius* is a sulphur metabolizing archaebacterium which grows at temperatures of 70-75°C and at a pH of 2-3. The starting material for the enzyme purification was a post-ribosomal cytoplasmic extract kindly provided by Dr. A. Matheson, U. of Victoria, B.C. Enolase was purified by a combination of ion. exchange chromatography on DEAE-cellulose (DE52) and gel filtration on Sephacryl S-300. The enzyme was characterized with respect to its native and subunit molecular weights. Parameters such as the K_m for 2-phosphoglycerate, pH optimum, magnesium dependence and heat stability were determined and compared with those of enolases from eubacteria and eukaryotes. Enolases from *S. acidocaldarius* and members of the eubacterial genus *Thermus* are unusual in that they are thermostable and octamers whereas enolases from other organisms are dimers.

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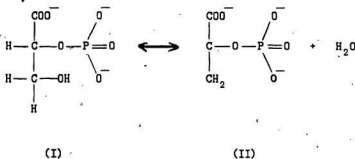
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Chapter 1

Introduction

1.1. Enolase

Enolase, 2-phospho-D-glycerate hydrolyase or phosphopyruvate hydratase (E.C. 4.2.1.11) is a central glycolytic enzyme which catalyzes the interconversion of 2-phosphoglycerate (2-PGA) (I) and phosphoenolpyruvate (PEP) (II). The reaction is as shown below:



As this reaction is part of the metabolic pathways of glycolysis, gluconeogenesis and fermentation, this enzyme is common to nearly all living species.

Enolase was first discovered by Lohmann and Meyerhof in 1934 and has

since been isolated and characterized from a large variety of biological sources which includes mammals (Wold, 1971), fish (Pietkiewicz *et al.*, 1983), plants (Miernyk and Dennis, 1984), yeast (Chin *et al.*, 1981) and prokaryotes (Spring and Wold, 1971; Stellwagen *et al.*, 1973; Barnes and Stellwagen, 1973). Enolase is present in all tissues of the rat (Kun, 1950). The enzyme has been partially purified from about twenty different sources and total purification has been obtained from nine of these sources.

Although extensive work has been done on yeast enolase, most of the well characterized enolases are from vertebrate muscles. Enolases are quite stable enzymes with the exception of ox brain enolase and potato enolase. The pure enzyme from ox brain enolase rapidly lost its activity as a result of undergoing changes in its structure and aggregation state (Wood, 1964). The enolase from potatoes seemingly has a very high content of sulphhydryl groups and is quite unstable (Boser, 1959).

1.2. Cation Requirement

Enolase is a metal-ion-activated enzyme in that it requires the presence of a divalent metal ion for activity. Several divalent metal ions such as zinc, manganese and magnesium can serve as activators (Wold, 1971). The cation requirements of the enzyme have shown that not only was the magnesium ion concentration required for maximum activity higher than that for zinc or manganese, but also that magnesium gave the highest maximal velocity of the activated reaction at a constant concentration of the enzyme and substrate (Pietkiewicz *et al.*, 1983). Therefore, it seems that the enzyme prefers magnesium

for maximum activity. The magnesium ion does not only play a part in the catalytic reaction, but it also has an important structural role in stabilizing the active dimeric form of the enzyme.

Magnesium activation studies on the enolase from *Escherichia coli* (Spring and Wold, 1971) have shown that the enzyme is unstable in the absence of magnesium ion and that when treated with ethylenediaminetetraacetic acid (EDTA) for a short period of time, there is a substantial and irreversible loss of activity of the enzyme. This denaturation of enolase in the absence of magnesium ion is not seen with other enolases, although in yeast and rabbit muscle enolase, removal of magnesium ions was found to greatly enhance dissociation of the dimer into inactive monomers (Brewer and Weber, 1968; Gawronski and Westhead, 1969; Winstead and Wold, 1965). Quantitative metal binding studies performed with yeast enolase have shown that in the absence of substrate, the enzyme binds at least two moles of magnesium ions. Additional magnesium ion binding sites become apparent in the presence of substrate or at high magnesium ion concentrations (Hanlon and Westhead, 1969).

1.3. Substrate Specificity

Enolase appears to be specific for 2-PGA and PEP as substrates. Detailed studies done on the specificity of the enzyme (Wold and Ballou, 1957b; 1959) have shown that of all the other substances tested (Table 1-1), not one was found to be a substrate for enolase, although some were able to act as inhibitors.

Table 1-1: Compounds tested as substrates for enolase

Compound	Inhibition
D-Lactic acid	None
D-Glyceraldehyde 3-phosphate	None
Dihydroxyacetone phosphate	None
β -Glycerol phosphate	None
β -Hydroxypropionic acid phosphate	Competitive
D-Lactic acid phosphate	Competitive
D-Glyceric acid 3- phosphate	Competitive
D-Erythryo 2,3-dihydroxybutyric acid 2-phosphate	Competitive
D-Erythryo 2,3-dihydroxybutyric acid 3-phosphate	Competitive
Inorganic phosphate	Non-competitive

1.4. Kinetic Properties

A comparison of some of the kinetic properties of enolases from different organisms have shown that the values of the Michaelis constant for the substrate, 2-PGA, are similar and range from $0.1 - 0.3 \times 10^{-3}$ M. Likewise the K_m s for PEP fall within the range of $0.6 - 0.9 \times 10^{-3}$ M. The pH optimum of *E. coli* enolase was found to be 8.1 (Spring and Wold, 1971) which is higher than that for vertebrate enolases (pH 7.0) and also yeast and plant enolases (pH 7.6 to 8.0).

1.5. Molecular Weight

Enolases from as widely different sources as yeast, fish and mammals consist of two identical or very similar subunits and have molecular weights within the range of 80,000 - 100,000. The catalytic site of enolase has remained quite stable throughout evolution. In *E. coli*, enolase exists as a dimer with a molecular weight of approximately 90,000 and a subunit molecular weight of $46,000 \pm 1,000$ (Spring and Wold, 1971).

Enolase does appear to have been preserved throughout evolution as dimers except in the case of the thermostable enolases isolated from two species of thermophilic bacteria in the genus *Thermus* (Stellwagen *et al.*, 1973; Barnes and Stellwagen, 1973). The enolase from the extreme thermophile *Thermus aquaticus* YT-1, was shown to be globular and to consist of eight apparently identical polypeptide chains each having a molecular weight of 44,000. Studies done on the enolase from the thermophilic bacterium *Thermus X-1* show the same results, except that the molecular weight of each polypeptide chain is 48,000. Instead of being dimers the enolases from members of the genus *Thermus* exist as octamers.

1.6. Multiple Forms of Enolase

The gene for enolase is constitutively expressed in both prokaryotes and eukaryotes (Fraenkel and Vinopal, 1973; Thomson *et al.*, 1970). In prokaryotes, only one form of the enolase has been found (Pfleiderer *et al.*, 1966; Spring and Wold, 1971), which is consistent with the *E. coli* chromosome containing a single genetic locus (*eno*) for enolase (Irani and Maitra, 1974; 1976). In contrast, eukaryotes possess at least two enolase genes each of which may be present in different organelles as in plants (Miernyk and Dennis, 1984) or may be expressed in different tissues (Wold, 1971). Yeast contains two nontandemly repeated enolase genes per haploid genome, which arose as a result of a recent gene duplication event. These genes are differentially expressed depending on the carbon source used to propagate the cells. It has also been shown that the random assortment of these two enolase polypeptides into active dimers results in the presence of three enolase isozymes in the cell (Holland *et al.*, 1981; McAlister and Holland, 1982). Similarly, mammalian tissues such as the liver and brain of rat show the presence of three types of enolase isozymes (Sakimura *et al.*, 1985). Multiple molecular forms have also been seen in some fish species (Tsuyuki and Wold, 1984; Cory and Wold, 1966; Ruth *et al.*, 1970).

1.7. 'Molecular Clock'

Comparison of the primary structures of homologous proteins from a range of species indicates that the amount of sequence divergence is proportional to the time since the organisms speciated. In other words, sequences change at nearly constant rates. From the divergence times of organisms, the rates of amino acid

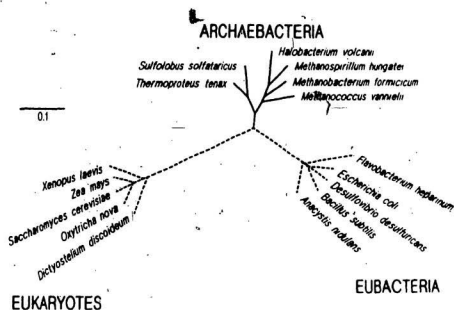
substitution of several proteins can be calculated. It has been shown that sequence evolution goes on at a constant rate for proteins within a given functional class, but that rates may differ for proteins having different functions (Wilson *et al.*, 1977).

The rate at which divergence has occurred can be measured as the percent amino acid sequence difference per million years, or as its reciprocal, the unit evolutionary period (UEP), i.e. the time in millions of years that it takes for a one percent divergence to develop between two lineages. This provides the basis for the 'Molecular Clock' that measures the accumulation of mutations at an apparently even rate during the evolution of a given protein (Dickerson and Geis, 1969).

1.8. The Three Primary Kingdoms

A phylogenetic analysis based upon ribosomal RNA (rRNA) sequences has indicated that living systems can be divided into three primary kingdoms. They are the eubacterial kingdom which consists of all typical bacteria and which can be further divided into the cyanobacteria, the gram-positive and the gram-negative bacteria; the archaeobacterial kingdom, comprising methanogens, thermoacidophiles and halobacteria; and the urkaryotic kingdom which is defined by structural similarities in the 18S rRNAs of the eukaryotic cytoplasm (Woese and Fox, 1977; Fox *et al.*, 1980). Figure 1-1 shows the three primary kingdoms as distinct phylogenetic units. This diagram was derived from a distance matrix-type analysis of the small subunit rRNAs.

Figure 1-1: The three primary kingdoms as distinct phylogenetic units



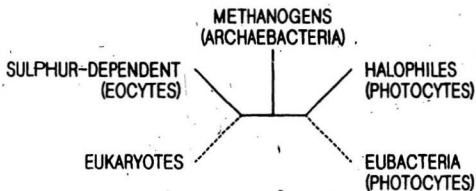
Recently there has been considerable controversy concerning the phylogenetic relationships among the archaeobacteria and eubacteria (Lake, 1986; Zillig, 1986; Woese *et al.*, 1986). Lake *et al.* (1985, 1986) claimed that the bacterial kingdom can be divided into three separate kingdoms namely the 'eocytes' which consist of the sulphur-dependent archaeobacteria like *Sulfolobus* and *Thermoproteus*; the 'photocytes' which are made up of the extreme halophiles and eubacteria; and the archaeobacteria comprising the methanogens (Figure 1-2). Lake *et al.* (1985, 1986) based their proposal on the variations of the three-dimensional ribosomal structure among the bacterial groups. They also claimed that the 'photocytes' are more closely related to one another than either is to members of the other kingdoms which include the eocytes, the remaining archaeobacteria (methanogens) and the eukaryotes.

Sequence comparisons of the ribosomal protein, HS3, from an archaeobacterium, *Halobacterium marismortui*, with those from other organisms have shown that some ribosomal components of the archaeobacteria are related to eubacteria and others to eukaryotes (Kimura and Lagner, 1984).

A comparison of the primary and secondary structures of the complete 16S and 16S-like rRNAs (18S) from representatives of the three primary kingdoms shows that the archaeobacterial 16S rRNA sequence is closer to the eubacterial 16S rRNA than it is to the eukaryotic 18S rRNA. On the other hand, these studies also showed that specific sequences of the archaeobacterial 16S rRNA were similar to that of its eukaryotic counterpart. These results therefore indicate that the sequence of the archaeobacterial 16S rRNA is closer to both the eubacterial and eukaryotic sequences than either is to one another (Gupta *et al.*, 1983).

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Figure 1-2: The distribution of the bacterial kingdom as seen by Lake
et al. (1985, 1986).



In contrast, other studies carried out on the comparisons of the amino-terminal sequences of ribosomal proteins from archaeobacteria with those from other organisms have shown that the archaeobacteria are more closely related to the eukaryotes than to the eubacteria. This implies, therefore, that the archaeobacteria diverged from eukaryotes after the divergence of the eubacteria and eukaryotes (Yaguchi *et al.*, 1982).

Since the results obtained from the comparative studies on ribosomal components are not sufficient to determine the phylogenetic origins of prokaryotes and eukaryotes, it is of importance therefore to study molecules other than ribosomal components.

Major phenotypic changes, such as the acquiring of a novel metabolic activity, depend initially on increasing the activity of a rate-limiting protein. In principle, this could be due to point mutations in the structural gene coding for the particular protein. However, studies show that the increase in enzyme activity is often brought about by other kinds of mutations. Among these are quantitative mutations which can result from point mutations in regulatory genes and genes that exert control at levels other than transcription. Chromosomal mutations that alter the arrangement of genes may also result in quantitative mutations. These mutations increase the effective concentration of the rate-limiting protein.

Since the molecular clock allows the properties of organisms to be easily discerned from a time perspective, the rates of evolutionary change can be calculated whether the properties are chromosomal or morphological.

The techniques of cloning genes and sequencing DNA, will reveal substitutions in genes that cannot be seen at the protein level. Hence these approaches may provide a sound method for studying all types of evolutionary changes.

Glycolytic enzymes evolve very slowly (Wilson *et al.*, 1977) and are therefore highly conserved. As the glycolytic enzymes involved in three-carbon metabolism are found in representatives of every phylogenetic lineage (Van Valen and Maiorana, 1980), these proteins and the genes that encode them are valuable tools for studying long range evolutionary relationships.

Chapter 2

Research Proposal

The purpose of this project was two-fold: First, as a start to comparing the *E. coli* enolase gene with its eukaryotic counterparts, it was important to locate the gene on a hybrid recombinant plasmid, pLC10-47, which was found to complement an *E. coli* enolase mutant and secondly, as there has been controversy over the phylogenetic relationships among the archaeobacteria and eubacteria, it was of interest to examine some properties of an enolase from a thermoacidophilic archaeobacterium, *Sulfolobus acidocaldarius*, and to compare these properties with those of enolases from the eubacterial thermophiles of the genus *Thermus* to determine if this protein shares any structural properties.

Chapter 3

Materials and Methods

3.1. Materials

Restriction endonucleases were purchased from Bethesda Research Laboratories (BRL) Inc., Gaithersburg, MD., USA. Agarose, lysozyme, ribonuclease, caesium chloride, ampicillin, the trisodium salts of 2-PGA and PEP, cytochrome c, chymotrypsinogen A, ovalbumin, chicken muscle lactate dehydrogenase, rabbit muscle enolase, yeast enolase and all other chemicals unless otherwise stated were bought from Sigma Chemical Co., St. Louis, MO., USA. Catalase and ferritin were obtained from Helix Biotech Ltd., Richmond, B.C. Diethylaminoethyl (DEAE) cellulose (DE52) and Phospho-cellulose (P-11) were Whatman products. Sephacryl S-300 and Blue dextran were purchased from Pharmacia (Canada) Ltd., Dorval, Que. Acrylamide, bisacrylamide, N,N,N',N'-tetramethylethylenediamine (TEMED), ammonium persulphate, DEAE ion exchange paper (DE81) and the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) low molecular weight standards (10,000 - 100,000) were obtained from BioRad (Canada) Ltd., Mississauga, Ont.

3.2. Strains and Plasmids

Cultures of *Escherichia coli* (JA200) and *E. coli* (JA200) containing pLC10-47 were obtained from Dr. Barbara J. Bachmann at the *E. coli* Genetic Stock Center, New Haven, CT., USA.

3.3. Growth Media and Buffers

Bacto-tryptone, bacto-agar and yeast extract were bought from British Drug House (Canada) Ltd., Halifax. The recipes for Luria-Broth (LB) and Luria-Broth/Ampicillin (LB/Amp) are given in Appendix I. The ingredients of standard buffers most commonly used such as 10 mM TE, 10X TBE, 10X TEA, reservoir buffer for polyacrylamide gel electrophoresis and others are also given in Appendix I.

3.4. Rapid Isolation of Plasmid DNA by the Miniscreen

Procedure

A 25 ml flask containing 10 ml LB or LB/Amp was inoculated with a single colony from cells containing the plasmid of interest. The culture was incubated overnight at 37°C with vigorous shaking. The cells were harvested by centrifugation in a bench-top centrifuge at top speed for five minutes and the supernatant discarded. Plasmid DNA was then isolated according to a modification (Maniatis *et al.*, 1982) of the rapid alkaline extraction procedure of Birnboim and Doly (1979). The plasmid DNA pellet was resuspended in 50 μ l of sterile 10 mM TE buffer and the purity of the DNA was checked by agarose gel electrophoresis.

3.5. Large Scale Isolation of Plasmid DNA

An overnight culture of *E. coli* containing the required plasmid was grown in 10 ml of LB or LB/Amp with vigorous shaking. 1 ml of the overnight culture was added to 250 ml of the appropriate media. The cells were allowed to grow at 37°C with vigorous shaking until the cell density reached an A_{550} of 0.5. Amplification of the plasmid was achieved by the addition of 2.5 ml of chloramphenicol (200 µg/ml) in ethanol. Incubation was continued for a further 12 - 16 hours. The cells were harvested by centrifugation and plasmid DNA isolated using the alkali/SDS lysis method of Maniatis *et al.* (1982).

The crude extract containing plasmid DNA was mixed with cesium chloride and ethidium bromide and subjected to equilibrium centrifugation to separate the supercoiled plasmid DNA from linear chromosomal DNA. The supercoiled plasmid DNA was removed from the tube by puncturing the side with a 21 gauge needle and drawing the DNA solution into a syringe. Ethidium bromide was removed from the DNA solution by extracting it with isoamyl alcohol saturated with water. Cesium chloride was then removed from the DNA solution according to the following protocol: for every 0.5 ml cesium chloride DNA solution, 1.1 ml of sterile water was added followed by 3.2 ml of 95% ethanol. Purified plasmid DNA precipitated out of solution at -20°C and was recovered by centrifugation. The plasmid DNA pellet was then resuspended in 200 µl TE. The purity of the DNA was checked by agarose gel electrophoresis.

3.6. Restriction Endonuclease Digestions

Samples of DNA containing the necessary restriction endonucleases were digested overnight at 37°C in final reaction volumes of 20 µl. Core buffer (supplied by the company) was used in all the digestions. The recognition sequences and the cleavage sites of the restriction endonucleases used in this research are given in Table 3-1

3.7. Agarose Gel Electrophoresis

Electrophoresis of DNA samples in 0.8% agarose gels was carried out in order to check the purity of plasmid samples and also to separate DNA fragments obtained as a result of the restriction endonuclease digestions. DNA fragments are separated on the basis of their size by gel electrophoresis. The sizes of the fragments can be determined by calibrating the gel by running a marker in a slot on the same gel. The marker contains a mixture of fragments all of known sizes. The migration of the markers defines the relationship between the size of the fragment and the distance moved for a particular gel. The most commonly used marker was λ DNA cleaved by Hind III. The buffer system used was TBE (see Appendix I).

After electrophoresis the gels were stained in 0.5 µg/ml ethidium bromide for thirty minutes and the DNA was visualized by ultra-violet (u.v.) transillumination (Ultra-violet Products, Inc., San Gabriel, California, USA). The gel was photographed using a Polaroid MP-4 camera with a Kodak Wratten 22A filter.

Table 3-1: Restriction endonucleases and their recognition sites and cleavage sites

Restriction Endonuclease	Recognition Site
Bam HI	G [*] GATCC
Bgl II	A [*] GATCT
Eco RI	G [*] AATTC
Hind III	A [*] AGCTT
Pst I	CTCGA [*] G
Sal I	G [*] TCGAC

* denotes the site at which the endonuclease cleaves

3.8. Recovery of Individual DNA Fragments

The fragment to be recovered was separated by electrophoresis in a 0.8% agarose preparative gel, stained with ethidium bromide and visualized by u.v. light. The buffer used was TEA (see Appendix I). A slit was made on the gel in front of the fragment to be recovered. A small strip of DE81 paper was placed in the slit and electrophoresis was allowed to proceed for twenty minutes. The gel was checked again under u.v. light to see if the DNA fragment had bound to the DE81 paper. The paper was then removed and placed in a 0.5 ml microfuge tube which had holes in the bottom of it. The tube was placed in a 1.5 ml microfuge tube and centrifuged at 12,000g for a minute to remove the TEA buffer. The small tube was then checked under u.v. light to see if the DNA was still bound to the paper.

100 μ l of buffer containing 1X TEA and 1 M NaCl at pH 8, was added to the paper in the 0.5 ml tube and left sitting over the 1.5 ml tube in a hot air incubator at 37°C for forty minutes. The tube was spun for two minutes at 12,000g and the solution in the 1.5 ml tube saved. The addition of buffer, incubation and removal of buffer was repeated two more times. The DE81 paper was checked under u.v. light to see that all the DNA had eluted from it. If necessary the washing off of the DNA from the DE81 paper with high salt was repeated. The resulting solutions containing the DNA were pooled together and ethidium bromide was removed by extracting with an equal volume of isobutanol. The tube was centrifuged at 12,000g for two minutes and the top layer containing the ethidium bromide in isobutanol was discarded. The DNA was precipitated

out of solution by the addition of two volumes of 95% ethanol and placing the tube overnight at -20°C . The DNA fragment was recovered by centrifugation at 12,000g for ten minutes at 4°C , discarding the ethanol and drying the pellet under vacuum. The pellet was resuspended in 20 μl TE and 1 μl of the DNA solution was subjected to gel electrophoresis to check for the recovery of the DNA.

3.9. Shotgun Cloning

14 μg each of the hybrid recombinant plasmid, pLC10-47 and the vector, pUC 12 was subjected separately to restriction endonuclease digestions. A sample of each digest was run on a gel to check if digestion was complete. An equal volume of phenol was added to each tube, mixed, allowed to stand at room temperature for five minutes and then centrifuged at 12,000g for two minutes. The upper aqueous layer of each tube was transferred to a separate clean microfuge tube and extracted again with an equal volume of chloroform:isoamyl alcohol (49:1). The upper aqueous layer was transferred to a separate clean microfuge tube and one-tenth the volume of 2 M sodium acetate, pH 5.1 was added to each tube. The DNA was precipitated out of solution by the addition of two volumes of cold 95% ethanol and left overnight at -20°C .

The DNA was recovered by centrifuging the tubes at 12,000g for forty-five minutes at 4°C , discarding the ethanol and drying the pellet under vacuum. The pellets were then resuspended in 10 μl of sterile water.

5 μl of the solution of DNA fragments from pLC10-47 was added to 1 μl of the vector. 1 μl of freshly made 10X ligation buffer (see Appendix I) and two

the vector. 1 μ l of freshly made 10X ligation buffer (see Appendix I) and two units of T_4 DNA ligase were added to the reaction mixture in a final volume of 10 μ l. The reaction mixture was incubated in a water-bath at 12°C for 12 - 16 hours. As a control the marker, λ DNA cleaved by Hind III, was allowed to undergo ligation. 1 μ l from each of the ligation mixtures was subjected to gel electrophoresis to check for ligated species.

3.10. Preparation of Frozen Competent Cells

Frozen competent *E. coli* DH1 cells were prepared using a modification of the procedure of Hanahan (1983). 5 ml of ϕ b (see Appendix I) was inoculated with a single colony of *E. coli* DH1 cells and incubated at 37°C with shaking until the cell density reached an A_{550} of 0.3. The culture was then transferred to 100 ml ϕ b and allowed to grow to an A_{550} of 0.48. The culture was chilled on ice for five minutes and centrifuged at 1,960g for fifteen minutes at 4°C. The cells were resuspended in 40 ml Tfb I (see Appendix I) and left on ice for five minutes. Cells were centrifuged at 1,960g for five minutes at 4°C and resuspended in 4 ml Tfb II (see Appendix I). The cells were left on ice for fifteen minutes and then aliquoted into 200 μ l samples in microfuge tubes and stored at -70°C. When required the cells were allowed to thaw out gently on ice and used as needed.

3.11. Transformation of Competent *E. coli* DH1 Cells

200 μ l of competent *E. coli* DH1 cells and 5 μ l of the ligated DNA species were added to a screw capped tube. The tube was placed on ice for an hour and then shaken gently in a 42°C water bath for ninety seconds. The tube was placed on ice for five minutes. 1 ml of LB was added to the tube and the contents were

gently mixed. The tube was incubated at 37°C, with gentle agitation, for an hour. The transformed cells were plated onto LB/Ampicillin plates and allowed to grow overnight at 37°C.

3.12. Preparation of Cell Extracts

E. coli was allowed to grow overnight in 10 ml LB or LB/Amp at 37°C with vigorous shaking. Crude extracts were prepared from *E. coli* (JA200 and DH1) alone and from *E. coli* (JA200) containing pLC10-47 and *E. coli* (DH1) containing pUC 12, pST 34, pST 34-E1 or pST 34-E2. 0.5 ml of the overnight culture was then transferred to a flask containing 125 ml LB or LB/Amp and the cells were allowed to grow at 37°C with vigorous shaking. When the A_{550} reached 0.5, cell growth was stopped by placing the flask on ice and 1 ml of cells was transferred to a clean tube and harvested by centrifugation. The supernatant was discarded and the pellet resuspended in 1 ml of breaking buffer (50mM Tris/HCl, pH 7.5 containing 10 mM $MgCl_2$). The cells were harvested again and resuspended in 1.5 ml of breaking buffer. Lysis of the cells was achieved by sonicating at 40 watts for 20 seconds using a Sonificer cell disruptor (Heat Systems, Ultrasonics Inc., Plainview, N.Y.). The cells were then cooled in ice water for one minute. The sonication and cooling steps were repeated four more times. The cell extract was then transferred to a clean 1.5 ml microfuge tube and centrifuged at 12,000g for 10 minutes at 4°C. The clear supernatant was transferred to a clean microfuge tube and used for enolase assays. The A_{280} of the cell extract was also determined and taken as a measure of the protein concentration.

3.13. Assay for Enolase Activity

Enolase activity was determined by measuring the rate of increase in absorbance at 240 nm due to the production of PEP using a Cary 118 recording spectrophotometer. The standard assay mixture contained 50 mM Tris base titrated to pH 6.5 with acetic acid, 10 mM $MgCl_2$ and 1 mM 2-PGA in a final volume of 1 ml. All assays were carried out at 25°C. Specific activities were calculated in terms of the change in absorbance at 240 nm per minute per ml of extract with an A_{280} of 1.

3.14. Purification of *S. acidocaldarius* Enolase

The starting material for the purification was a freeze-dried post-ribosomal cytoplasmic extract of *Sulfolobus acidocaldarius*, strain 98-3 (Brock, 1985) that was kindly provided by Dr. A. T. Matheson of the Department of Microbiology and Biochemistry, University of Victoria, Victoria, British Columbia. 3.2 g of the freeze-dried extract was resuspended in 50 ml of ice-cold water and dialysed overnight against 4 l of Buffer A (20 mM Tris/HCl, pH 8.0 containing 1 mM $MgCl_2$) at 4°C. The membrane used for dialysis was a standard cellulose dialysis tubing with a molecular weight cut off of 12,000 - 14,000.

The dialysed extract was then applied to a column (1.5 x 15 cm) of DEAE-cellulose (DE52) which had previously been equilibrated with Buffer A. The column was washed successively with 200 ml of Buffer A, 200 ml of Buffer A containing 0.1 M NaCl, 200 ml Buffer A containing 0.25 M NaCl and finally 200 ml Buffer A containing 1 M NaCl. Fractions were checked for enolase activity using the standard assay and protein was measured by reading the absorbance at

280 nm. Enolase activity was found in the fractions eluted with 0.25 M NaCl. The fractions were pooled and concentrated to a volume of 5 ml using an Amicon ultrafiltration unit and a Diaflo Ultrafiltration YM-10 membrane. The concentrated protein was then applied to a column of Sephacryl S-300 (1 x 110 cm) in Buffer A. Fractions containing activity were pooled and applied directly to a DEAE-cellulose column (1.5 x 15 cm) equilibrated with Buffer A. This column was washed with 100 ml of Buffer A, then Buffer A containing 0.1 M NaCl and then subjected to a linear gradient of 0.1 - 0.3 M (0.5 mM/ml) NaCl in Buffer A. The most active fractions were pooled, concentrated as above to a volume of 1 ml and applied to a column of Sephacryl S-300 (1 x 110 cm) in Buffer A. This gel filtration step was used to purify the enolase further and also to determine the native molecular weight of the enolase from *S. acidocaldarius*. The active fractions from the Sephacryl S-300 column were used to characterize the enzyme.

For the purpose of determining the subunit molecular weight the *S. acidocaldarius* enolase, the enzyme was further purified. The active fractions obtained as a result of the second DEAE-cellulose column were pooled, concentrated to a volume of 5 ml and dialysed overnight against 4 l of Buffer B (20 mM Tris/HCl, pH 6.5 containing 1 mM $MgCl_2$) at 4°C. The dialysed fraction was applied to a column (1.5 x 15 cm) of phospho-cellulose (P-11) in Buffer B. This column was washed successively with 100 ml of Buffer B and 100 ml of Buffer B containing 1 M NaCl. Active fractions eluted with high salt were pooled, concentrated and applied onto a Sephacryl S-300 column (1 x 110 cm) in Buffer A. The active fractions from this last purification step were pooled,

concentrated and used to determine the subunit molecular weight of the enzyme. All purification procedures were carried out at 4°C. The purification steps are summarized in Figure 3-1.

3.15. Molecular Weight Determination

3.16. Gel Filtration

The native molecular weight of *S. acidocaldarius* enolase was determined by gel filtration on Sephacryl S-300 as described above. The column was calibrated with Blue dextran (void volume), ferritin (440,000), catalase (232,000), chicken lactate dehydrogenase (140,000), rabbit muscle enolase (82,000), ovalbumin (45,000) and cytochrome c (12,000).

3.17. SDS Polyacrylamide Gel Electrophoresis

The subunit structure of the enolase from *S. acidocaldarius* was investigated by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS). 10% Polyacrylamide slab gels (20 cm in length) were used according to the procedure of Laemmli (1970). An equivalent of 25 µg and 50 µg of protein from the purified extract of *S. acidocaldarius* was freeze-dried overnight using a Labconco freeze dryer. The freeze-dried samples were taken up in 5 µl of 1X SDS buffer and heat treated in a boiling water-bath for three minutes. Samples were cooled at room temperature and loaded on the SDS gel. A set of proteins consisting of the α and β chains of insulin (3,000), bovine trypsin inhibitor (6,200), lysozyme (14,300), β-lactoglobulin (18,400), chymotrypsinogen A (25,700) and ovalbumin (43,000) was used as molecular

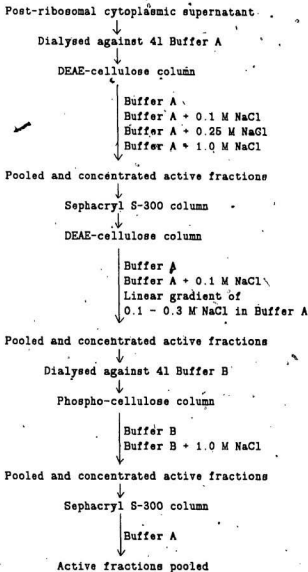


Figure 3-1: Purification steps of *S. acidocaldarius* enolase

weight markers. Yeast enolase (46,500) was also used. The gels were stained with 0.1% Coomassie Blue R-250 in a solution containing water/methanol/acetic acid (65/25/10) for one hour and then destained with several changes of 7% acetic acid.

3.18. Determination of the Extinction Coefficient of PEP

The extinction coefficient of PEP was determined at pH values increasing in steps of 0.5 pH units, between pHs 4.5 and 9.0. The assay medium contained 50 mM Tris base titrated to the required pH with acetic acid, 10 mM $MgCl_2$ and 1 mM PEP in a final volume of 1 ml. Assays were also done at different pHs in the presence and absence of $MgCl_2$ and/or PEP. All assays were carried out at 25°C. The absorbance at 240 nm was measured. The extinction coefficient in $mM^{-1} cm^{-1}$ for PEP was calculated based on the Beer-Lambert law expressed as $A = E \cdot c \cdot l$ where A = absorbance of the assay medium, E = a constant termed as the extinction coefficient, c = millimolar concentration of the absorbing component and l = length of the light path in solution (usually 1 cm).

3.19. Effect of pH on Enolase Activity

The optimum pH for maximum activity of enolase was determined by carrying out enzyme assays in Tris/acetate buffers at pHs ranging from 5.0 - 9. The assay medium consisted of 50 mM Tris base titrated to the required pH with acetic acid, 10 mM $MgCl_2$ and 1 mM 2-PGA in a final volume of 1 ml. All assays were carried out at 25°C. The final pH of the reaction mixture was also measured.

3.20. Magnesium Ion Concentration

The effect of magnesium ion concentration on the activity of the enolase from *S. acidocaldarius* was investigated by carrying out enzyme assays at different magnesium ion concentrations ranging from 0.5 mM - 20 mM. The assay medium contained 50 mM Tris/acetate, pH 6.5, 1 mM 2-PGA and the required magnesium ion concentration in a final volume of 1 ml. All assays were carried out at 25°C.

3.21. Substrate Concentration

The effect of substrate concentration on the activity of the enzyme was also observed by varying the concentration of the substrate, 2-PGA, in the assay medium from 0.005 - 2.0 mM. The other contents of the assay medium were 50 mM Tris/acetate, pH 6.5 and 1 mM $MgCl_2$. All assays were carried out at 25°C.

3.22. Heat Denaturation Studies

The thermal stabilities of rabbit muscle enolase and the enolase from *S. acidocaldarius* were determined by the procedure of Stellwagen *et al.* (1973). Samples of the enzyme from rabbit muscle enolase and *S. acidocaldarius* enolase were heated, for five minutes, at different temperatures ranging from 30°C to 95°C. Samples were then cooled in an ice-water bath for fifteen minutes and assayed at 25°C for enolase activity using the standard assay medium. Enolase activity was also measured for samples left at room temperature.

Chapter 4

Cloning of the *E. coli* Enolase Gene

4.1. Background

Two hybrid recombinant plasmids, pLC10-47 and pLC11-8, isolated from the Clarke-Carbon *E. coli* genomic clone bank (Clarke and Carbon, 1976) were found to complement a strain of *E. coli* with a mutation in its enolase gene (Thomson *et al.*, 1979). As a start to comparing the structure of the *E. coli* enolase gene with its yeast counterpart, it was important to clone the gene first. For the purpose of this project the hybrid recombinant plasmid, pLC10-47, was selected because pLC11-8 did not exhibit any enolase activity. It is possible that the pLC11-8 obtained from the *E. coli* Genetic Stock Center is not the same as the pLC11-8 described by Thomson *et al.* (1979) (Looi-Lyons, 1987).

The restriction endonuclease map for the hybrid recombinant plasmid, pLC10-47, was constructed by digesting the plasmid with six base recognition enzymes in order to identify and map the restriction endonuclease sites on the plasmid. The location of the cloning vector, Col E1, in the plasmid was determined by comparing the restriction maps of pLC10-47 and Col E1 and also by Southern blot analyses using Col E1 as the radioactively labelled probe (Figure 4-1). The approximate size of this plasmid is 20.0 kilobase pairs (kbp). The

characterization of the recombinant plasmid, pLC10-47, was carried out by Looi-Lyons (1987).

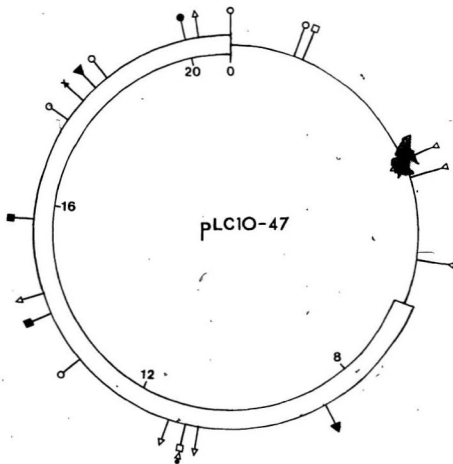
4.2. Approach of Investigation

In order to narrow down the location of the enolase gene in the hybrid recombinant plasmid, pLC10-47, it was decided to subclone fragments of the plasmid first and determine which of the subclones contained the gene. This procedure involved cutting the plasmid with restriction endonucleases to give smaller fragments and ligating these fragments to a plasmid vector. Host cells were then transformed with the ligation mix and the DNA was isolated from these transformants. The DNA was linearized by cutting with a single restriction enzyme, and samples were run on a 0.8% agarose gel to see if they contained any inserts. Finally the cell extracts of the transformants were assayed to check for any increase in enolase activity. Since the gene for enolase is constitutively expressed it is assumed that any increase in enzyme activity will be due to a gene dosage effect, i.e. extra genes present on the plasmid.

Figure 4-1:

Restriction map of the plasmid, pLC10-47.

The thin line represents the cloning vector and the boxed region represents the *E. coli* DNA insert. The symbols for the restriction enzyme sites are as follows: Bam HI (⌈), Bgl I (⌈), Bgl II (⌈), Eco RI (⌈), Hind III (⌈), Kpn I (⌈), Pst I (⌈), Pvu II (⌈) and Sma I (⌈).



4.3. Results

4.3.1. Subcloning

Restriction endonucleases recognize specific nucleotide sequences in a segment of DNA and cleave the DNA into a number of smaller fragments. The number of fragments generated depends on the number of recognition sites for the enzyme present in the DNA. Digestion of purified pLC10-47 (from large scale preparation) with Bgl II is expected to give a single fragment. Digestion of the plasmid with Hind III and Bam HI will each give two fragments. Triple digestion of pLC10-47, with restriction endonucleases, Bgl II, Hind III and Bam HI yielded five fragments varying in size from 1.8 to 9.0 kbp (Figure 4-2).

The vehicle used for subcloning and amplification of the plasmid DNA fragments was pUC 12 (Figure 4-3). This plasmid vector of size, 2.7 kbp, has the advantage of having a multiple cloning site. The cloning vector, pUC 12, also has a β -lactamase gene which confers ampicillin resistance to the vector. This, therefore, allows for selection of the transformants when the cells are plated onto media containing ampicillin.

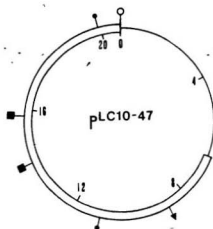
The vector, pUC 12, was digested with restriction endonucleases, Bam HI and Hind III, to produce a linear fragment and a very small fragment. It is important to note that the recognition sites of the restriction endonucleases, Bgl II and Bam HI are compatible. Figure 4-4 gives an illustration of this compatibility.

By the process of shotgun cloning the fragments from pLC10-47 were ligated to pUC 12. Frozen competent *E. coli* DH1 cells were transformed with the

Figure 4-2:

Digestion of pLC10-47 with Bgl II, Hind III and Bam HI.

The symbols for the restriction enzyme sites are: Bam HI (⌈),
Bgl II (⌈) and Hind III (⌈).



Bgl II / Hind III / Bam HI

	kbp
Bgl II — Hind III	≈2.5
Hind III — Bam HI	≈2.9
Bam HI — Bam HI	≈1.8
Bam HI — Hind III	≈4.3
Hind III — Bgl II	≈8.8

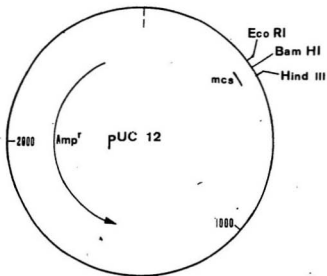
Figure 4-3:

Restriction map of pUC 12.

Amp^r: ampicillin resistance region

mcs: multiple cloning site





Bgl II

AGATCT
TCTAGA

Bam HI

GGATCC
CCTAGG

GATCT GATCC
 A G
 A G
 TCTAG CCTAG

GGATCT
CCTAGA

AGATCC
TCTAGG

Figure 4-4: Compatibility of the Bgl II and Bam HI sites.

ligation mix and plated onto LB/Amp plates for selection of transformants (Figure 4-5).

4.3.2. Screening of Transformants

To check for inserts the plasmid DNA from the transformants was isolated using the miniscreen procedure. The DNA was linearized by cutting it with a single restriction enzyme, Hind III. A sample of the linearized DNA was then electrophoresed on a 0.8% agarose gel. λ DNA cleaved with Hind III was run alongside the DNA samples in order to find out the size of the plasmid DNA. Plasmid DNA containing an insert will be bigger than that of the pUC 12 vector itself. Knowing that the size of the vector is 2.7 kbp, the size of the insert can be obtained. Linearized plasmids obtained from transformants were grouped together based on the size of the plasmids and therefore the possible inserts they might contain. Table 4-1 gives a list of these transformants. The smallest fragment, Bam HI-Bam HI, has only one end that is compatible with the linearized pUC 12 vector, hence ligation of this insert to the vector was not expected to take place. Indeed, no such recombinant was found. However, the gene for enolase is not expected to be contained in this fragment due to the fact that the minimum size for the coding region of the enolase gene is approximately 1.4 kbp and the size of the Bam HI-Bam HI fragment is about 1.8 kbp.

Cell extracts of these transformants were assayed to see if there was any increase in enolase activity. This would give an indication of whether or not the particular transformant contained the gene for enolase. Table 4-2 shows the results of the enolase assays. Please note that transformants in Group E contained the vector only and therefore no increase in enolase activity is expected.

Figure 4-5:

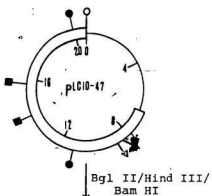
Flow diagram of the transformation of *E. coli* DH1 cells.

The symbols for the restriction enzyme sites are: Bam III (¶),

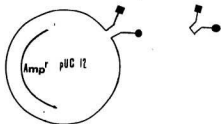
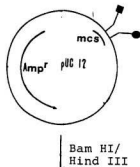
Bgl II (†) and Hind III (‡).

Amp^r: ampicillin resistance gene

mcs: multiple cloning site.



Bgl II — Hind III
Hind III — Bam HI
Bam HI — Bam HI
Bam HI — Hind III
Hind III — Bgl II



Mix, add T₄ DNA ligase

At 12⁰C for 12 - 16 hours

Transform E. coli DH1 cells

LB/Ampicillin plates

Select transformants

Table 4-1: Groups of plasmids obtained after transforming *E. coli* DH1

Group	Transformant	Linearized Plasmid	Insert Size	Probable Insert
A	pST 1	5.2	2.5	Bgl II-Hind III
	pST 10	5.2	2.5	Bgl II-Hind III
B	pST 5	10.9	8.2	Hind III-Bgl II
	pST 8	11.0	8.3	Hind III-Bgl II
C	pST 34	7.1	4.4	Bam HI-Hind III
	pST 38	7.2	4.5	Bam HI-Hind III
D	pST 35	6.1	3.3	Hind III-Bam HI
	pST 45	5.9	3.1	Hind III-Bam HI
E	pST 12	2.9	-	-
	pST 18	2.7	-	-

Table 4-2: Comparison of enolase activities in groups of transformants

Group	Transformant	$A_{240} \text{ min}^{-1}/\text{ml}$	A_{280}/ml	$A_{240} \text{ min}^{-1}/A_{280}$
A	pST 1	0.060	0.940	0.064
	pST 10	0.070	0.960	0.073
B	pST 5	0.072	1.160	0.062
	pST 8	0.061	0.800	0.076
C	pST 34	0.402	0.700	0.574
	pST 38	0.042	0.580	0.072
D	pST 35	0.059	0.940	0.063
	pST 45	0.078	1.020	0.077
E	pST 12	0.088	1.160	0.076
	pST 18	0.108	1.340	0.081

4.3.3. Transformant pST 34

The cell extract of one transformant, pST 34, was found to have an increase in enolase activity which was about six times higher when compared to the enolase activities of other transformants. The plasmid DNA isolated from the transformant, pST 34, when linearized with Hind III, was found to be 7.1 kbp in length. Knowing the size of the pUC 12 vector to be approximately 2.7 kbp, this implied that the insert subcloned was about 4.4 kbp which therefore indicated that the insert was the larger Bam III-Hind III fragment (Figure 4-6).

To confirm the presence of the Bam III-Hind III insert, the purified plasmid DNA of pST 34 was subjected to a series of single and double digests. The restriction map of the parent plasmid, pLC10-47 (see Figure 4-1), shows that the 4.4 kbp Bam III-Hind III fragment contains a single Eco RI site and two Pst I sites. Studies had shown that pLC10-47 contains four Sal I sites although these sites were not mapped (Loqi-Lyons, personal communication). The results obtained from cleaving plasmid pST 34 with various restriction enzymes are shown in Figure 4-7.

The Sal I site of the pUC 12 vector had been removed from the multiple cloning site when the vector was digested with Bam III and Hind III simultaneously. When the plasmid DNA of pST 34 was digested separately with restriction enzymes Sal I and Hind III, a linearized fragment was obtained in each case which was 7.1 kbp. A double digest of pST 34 with Sal I and Hind III gave a larger fragment (4.5 kbp) and a smaller fragment (2.7 kbp), thus indicating the presence of a Sal I site within the insert. Double digest of pST 34 with Bam III

Figure 4-6:

Restriction map of transformant pST 34.

The symbols for the restriction enzyme sites are: Bam HI (T),
Eco RI (T), Hind III (T) and Pst I (T).

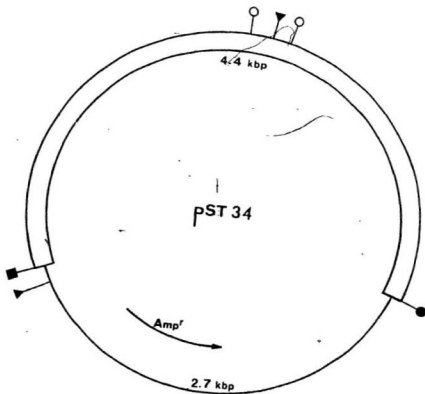
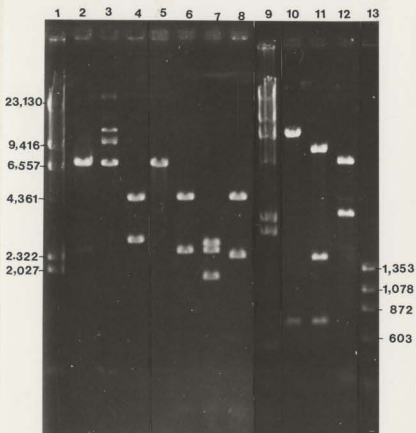


Figure 4-7:

Restriction endonuclease analysis of pST 34.

Lanes 1 and 9 corresponds to λ DNA cleaved by Hind III. Lanes 2-8 and 10-12 correspond to the digestions of pST 34 with: (2) Sal I (3) Hind III (4) Sal I/Hind III (5) Bam HI/Sal I (6) Eco RI (7) Eco RI/Hind III (8) Eco RI/Sal I (10) Pst I (11) Pst I/Hind III (12) Pst I/Eco RI. Lane 13 corresponds to ϕ X174 DNA cleaved with Hae III.



and Sal I yielded a large fragment, approximately 7.1 kbp, which further indicates that the Sal I site contained within the insert is close to the Bam HI site of the insert.

Since there is an Eco RI site near the Bam HI site of pUC 12 and another Eco RI site near the centre of the insert, digestion of pST 34 with Eco RI can be expected to yield two fragments, one of which will contain the vector and part of the insert (4.6 kbp). The results obtained with the restriction enzyme digest of pST 34 with Eco RI confirmed this. A double digest of pST 34 with Eco RI and Hind III gave three fragments of the order of 2.65 kbp, 2.45 kbp and 1.90 kbp respectively which is the same as the expected result. As the previous results indicated a Sal I site within the insert, double digestion of pST 34 with Eco RI and Sal I is expected to give three fragments, one of which will be too small to be seen on the gel. The results obtained were as expected.

Since there are two Pst I sites close to one another within the insert, digestion of pST 34 with Pst I is expected to give a larger fragment of the order of 6.5 kbp and a much smaller fragment of the order of 0.6 kbp. Double digests of pST 34 with Hind III/Pst I and with Pst I/Eco RI are expected to yield three fragments in the former digest and four fragments in the latter digest. As two of the fragments in the latter digest are very small in size, they will not be seen on the agarose gel. Again, the results obtained of the restriction enzyme digests of pST 34 are the same as the expected results confirming that the insert contained in the plasmid pST 34 is indeed the 4.4 kbp Bam HI-Hind III fragment.

In order to narrow down the location of the enolase gene contained on the plasmid pST 34, the purified plasmid was digested with the restriction endonuclease Eco RI (Figure 4-8) to obtain a larger fragment containing the vector and part of the insert (4.7 kbp) and a smaller fragment which was the remaining part of the insert (2.4 kbp). The fragments obtained were denoted by E1 and E2 respectively. Individual fragments were recovered by using DE81 paper and gel electrophoresis. Figure 4-9 shows the successful recovery of the individual fragments, E1 and E2. Fragment E1 was self-ligated using T₄ DNA ligase. Frozen competent *E. coli* DH1 cells were transformed with the ligation mix and plated onto LB/Amp plates for selection of transformants. Fragment E2 was ligated to pUC 12 which had been cut with Eco RI and the 5' ends dephosphorylated using calf intestinal phosphatase to minimize self ligation of the vector. Frozen competent *E. coli* DH1 cells were transformed with the hybrid plasmid and plated onto LB/Amp plates for selection of transformants.

Transformants were screened by examining the plasmid DNA they contained. As a result, transformants pST 34-E1 and pST 34-E2 containing fragments E1 and E2 respectively were isolated. The confirmation of the structures of these plasmids is described in sections 4.3.3. and 4.3.4.

Figure 4-8:

Digestion of pST 34 with Eco RI.

The symbols for the restriction enzyme sites are: Bam HI (⌈),

Eco RI (⌋), Hind III (⌈) and Pst I (⌋).

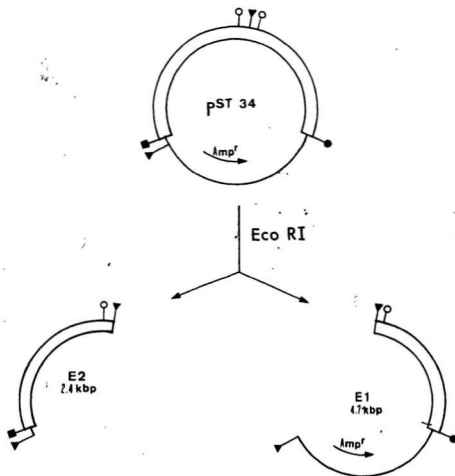
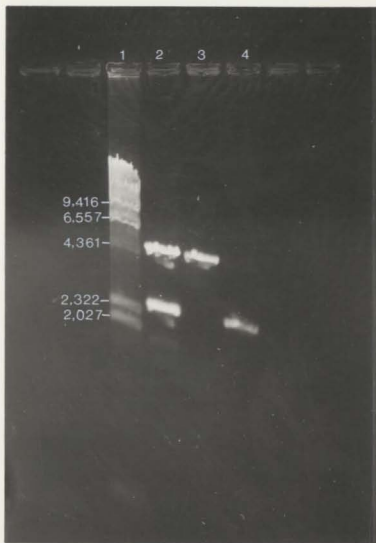


Figure 4-9:

Recovery of fragments E1 and E2.

The lanes correspond to the following: (1) λ DNA/Hind III (2)
pST 34/Eco RI (3) E1 and (4) E2.



4.3.4. pST 34-E1

The transformant pST 34-E1, was subjected to a single digest with Eco RI and a double digest with Eco RI and Hind III. With the single digest, only one linearized fragment, approximately 4.6 kbp is expected since the fragment E1 containing only one Eco RI site had been self-ligated. With the double digest two fragments were expected, an Eco RI-Hind III fragment (about 2.0 kbp) and the vector pUC 12 itself. Figure 4-10 shows that the results obtained were as expected.

The cell extract of pST 34-E1 was then assayed for enolase activity to see if there was any increase in activity. The results showed no increase in enolase activity indicating that it either did not contain the gene for enolase or that the gene or some regulatory region of the gene had been inactivated when the plasmid pST 34 was digested with Eco RI.

4.3.5. pST 34-E2

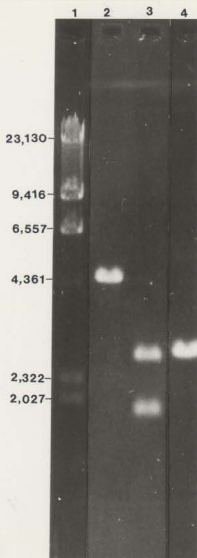
With the subcloning of the E2 fragment there could be two possible orientations of the fragment itself when ligated to the vector. Either the fragment E2 could orient itself in such a way that the Bam HI site of the fragment is furthest away from the Bam HI, Pst I and Hind III sites of the pUC 12 vector or it could orient itself so that the Bam HI site is very close to the above named sites of the vector (See Figure 4-11 and 4-13)

If the fragment was in the former orientation, then single digests with Bam HI or Eco RI would produce two fragments each of which is about 2.0 kbp in size.

Figure 4-10:

Restriction enzyme digestion of pST 34-E1.

The lanes correspond to the following: (1) λ DNA/Hind III (2) pST 34-E1/Eco RI and (3) pST 34-E1/Eco RI/Hind III and (4) pUC 12/Eco RI.



A single digest with Hind III would give a linearized fragment which would be equal to the size of the plasmid and another single digest with Pst I would give a larger fragment and a much smaller fragment which will not be seen on the gel, as it will be a few base pairs in length (Figure 4-11 and Figure 4-12).

On the other hand if the fragment was in the latter orientation, then single digests with Eco RI or Pst I would yield two fragments each of the magnitude of 2.0 kbp in length. A single digest with Hind III would give a linearized fragment as in the case of the former orientation and a single digest with Bam III would give a larger fragment and a much smaller fragment which will not be seen on the gel (Figure 4-13 and Figure 4-14).

However, when pST 34-E2 was subjected to single restriction enzyme digests with Hind III, Bam III, Eco RI and Pst I, the results obtained did not give any of the expected results (Figure 4-15). With Hind III, two fragments were obtained instead of one, a larger fragment of the order of 4.0 kbp and a smaller intense fragment which was about 2.45 kbp. With Bam III, two fragments were seen, both of which co-migrated with the Hind III fragments. With single digests of Eco RI and Pst I, two fragments each were obtained. In the last two cases, the large fragments showed up as a very intense band and was approximately 2.45 kbp in length. The other fragment was slightly smaller than the intense band.

The results obtained with the restriction enzyme digests of pST 34-E2 can be explained if three pieces of DNA ligated together i.e. the fragment E2 was ligated to two pUC 12 vectors (Figure 4-16) and the orientation of the insert being

Figure 4-11:

The first possible orientation of the insert contained within pST 34-E2.

The symbols for the restriction enzyme sites are: Bam HI (⌈), Eco RI (⌈), Hind III (⌈) and Pst I (⌈).

Figure 4-12:

Expected results of the restriction enzyme digestion of pST 34-E2 with the first possible orientation.

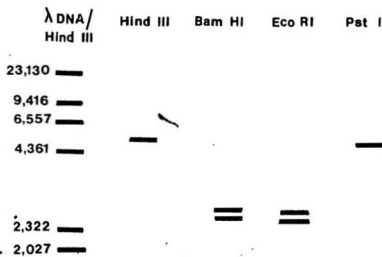
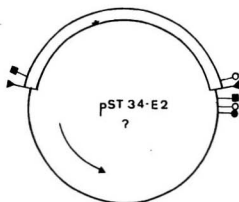


Figure 4-13:

The second possible orientation of the insert contained within the pST 34-E2.

The symbols for the restriction enzyme sites are: Bam HI (⌈), Eco RI (⌈), Hind III (⌈) and Pst I (⌈).

Figure 4-14:

Expected results of the restriction enzyme digestion of pST 34-E2 with the second possible orientation.

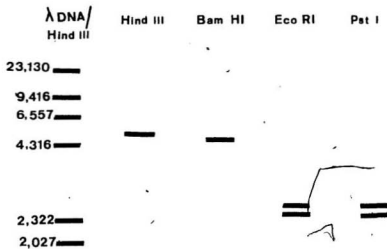
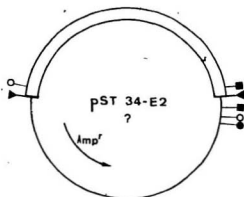


Figure 4-15:

Restriction enzyme digestion of pST 34-E2.

The lanes correspond to the following: (1) λ DNA/Hind III (2) uncut pST 34-E2 (3) pST 34-E2/Hind III (4) pST 34-E2/Bam HI (5) pST 34-E2/Eco RI and (6) pST 34-E2/Pst I.



such that the Bam HI site of the insert is closest to the Bam HI, Pst I and Hind III sites of one of the vectors. These results also explain the intensities of the bands obtained. After a sample of DNA is run on an agarose gel, the gel is stained with ethidium bromide. Ethidium bromide intercalates DNA and the amount of staining is proportional to the amount of DNA present which in turn is proportional to the intensity of the band when viewed under u.v. light. If there are indeed two or more vectors joined together then when the parent plasmid is cut with appropriate enzymes the band on the gel due to the vectors will show up as a very intense band which is what the results show.

4.3.6. Removal of the "Extra" pUC 12 Vector

To confirm that pST 34-E2 did contain two pUC 12 vectors and the fragment E2 ligated together, the plasmid was subjected to a single restriction enzyme digest with Hind III in order to remove the "extra" vector. Individual fragments were recovered by using DE81 paper and gel electrophoresis. Samples of each fragment were then run on a 0.8% agarose gel to check for recovery. Two fragments, 4.6 kbp and 2.5 kbp in length were obtained. The fragments were denoted as E2-A and E2-B respectively (Figure 4-17).

Fragment E2-A was then self-ligated using T₄ DNA ligase. Frozen competent *E. coli* DH1 cells were transformed with the ligation mix and plated onto LB/Amp plates to select for transformants. Plasmid DNA was isolated from transformant pST 34-E2-A and subjected to a series of single restriction enzyme digests. Figure 4-18 shows the results of these digests. With either Hind III or Bam HI, a large fragment of approximately 4.65 kbp was seen. The Eco RI digest

Figure 4-16:

Actual restriction map of pST 34-E2.

The symbols for the restriction enzyme sites are: Bam III (⌈),
Eco RI (⌋), Hind III (⌋) and Pst I (⌋).

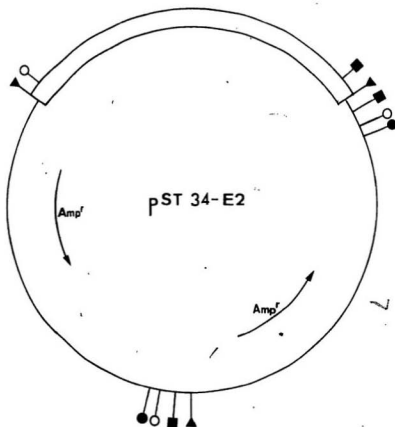
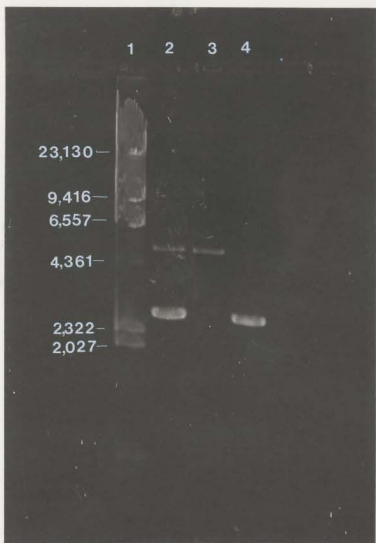


Figure 4-17:

Recovery of fragments E2-A and E2-B.

The lanes correspond to the following: (1) λ DNA/Hind III (2)

pST 34-E2/Hind III (3) E2-A and (4) E2-B.



gave two smaller fragments, 2.5 kbp and 2.3 kbp in length and the Pst I digest also resulted in two smaller fragments of the order of 2.6 kbp and 2.15 kbp. These results are comparable with the expected results discussed earlier and shown in Figure 4-13. These results also confirm the orientation of the E2 fragment in that it is ligated to the vector in such a way that the Bam HI site of the fragment is closest to the Bam HI, Pst I and Hind III sites of the vector. This would also indicate that the digest with Bam HI actually gave two fragments, the other one being too small to be seen on the gel.

4.3.7. Confirmation of a Sal I site.

As mentioned earlier, the parent plasmid, pLC10-47, was found to contain four Sal I sites which had not been mapped. The results obtained so far have shown one of the Sal I sites to be present within the 4.4 kbp Bam HI-Hind III insert.

When pST 34 was cut with Hind III and Sal I simultaneously, two fragments were obtained, i.e., a 4.4 kbp fragment (HS1) and a 2.7 kbp fragment (HS2). The large fragment was almost the size as the insert, Bam HI-Hind III, in the plasmid pST 34 indicating that the Sal I site must be near the Bam HI site. Individual fragments were then isolated (Figure 4-19) and subjected to a single restriction enzyme digest with Eco RI. Figure 4-20 shows the results of this digest. The large fragment, HS1, when cut with Eco RI gave two fragments, 2.4 kbp and 1.85 kbp. If the Sal I site is close to the Bam HI site of the insert then digestion with Eco RI will give two fragments, as seen above, since there is an Eco RI site within the insert.

Figure 4-18:

Restriction enzyme digest of pST 34-E2-A.

The lanes correspond to the following: (1) λ DNA/Hind III (2) pST 34-E2-A/Hind III (3) pST 34-E2-A/Eco RI (4) pST 34-E2-A/Bam III and (5) pST 34-E2-A/Pst I.

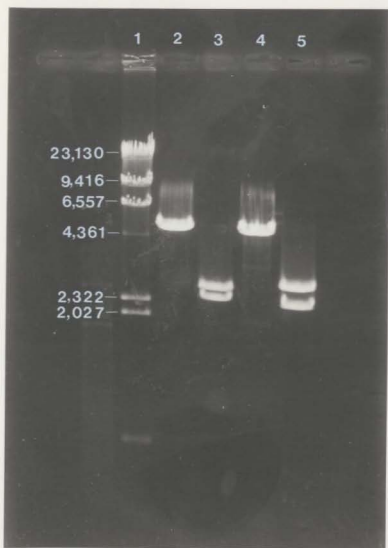


Figure 4-19:

Recovery of fragments HS1 and HS2

The lanes correspond to the following: (1) λ DNA/Hind III (2)
pST 34/Hind III/Sal I (3) HS1 and (4) HS2.



Figure 4-20:

Restriction enzyme digest of HS1 and HS2 with Eco RI.

The lanes correspond to the following: (1) λ DNA/Hind III (2) HS1 (3) HS1/Eco RI (4) HS2 and (5) HS2/Eco RI.



Treatment of the smaller fragment, HS2, with Eco RI, should then give a large fragment and a very small fragment which will not be seen on the gel as it is a only few base pairs long. The results obtained with the digestion of fragment HS2 with Eco RI was in agreement with the expected results. This therefore confirms the presence of a Sal I site close to the Bam HI site of the insert contained in the plasmid pST 34.

4.3.8. Comparison of Enolase Activities from Host Cells and Host Cells containing Plasmids and the Vector

Cell extracts from the host cells, *E. coli* (JA200 and DH1) and from *E. coli* JA200 containing pLC10-47 and also from *E. coli* DH1 containing pUC 12, pST 34, pST 34-E1 or pST 34-E2 were each assayed for enolase activity. Table 4-3 shows a comparison of the results obtained. As is seen, both the parent plasmid, pLC10-47 and the transformant pST 34 showed the highest increase in enolase activity (about 10 fold) when compared to the amount of enolase activity in the other cells. These results confirm yet again the successful cloning of the enolase gene into pST 34.

4.4. Discussion

Multiple cellular forms of enolase have been observed in a wide variety of eukaryotic cells. The existence of enolase isozymes in yeast was first described by Malmstrom (1957) and has since been investigated by other scientists (Westhead and McLain, 1964; Pfleiderer *et al.*, 1968; McAlister and Holland, 1982).

Yeast contains two non tandemly repeated enolase structural genes per

Table 4-3: A comparison of enolase activities from host cells and host cells containing plasmids

Sample	$A_{240} \cdot \text{min}^{-1} / \text{ml}$	A_{280} / ml	$A_{240} \cdot \text{min}^{-1} / A_{280}$
JA200	0.263	2.150	0.122
JA200/pLC10-47	2.954	2.800	1.055
DH1	0.198	1.79	0.111
DH1/pUC 12	0.121	1.168	0.104
DH1/pST 34	0.929	0.960	0.967
DH1/pST 34-E1	0.408	2.080	0.196
DH1/pST 34-E2	0.312	2.100	0.149

haploid genome (Holland *et al.*, 1981). Both these enolase genes are expressed and their loci have been termed Eno 1 and Eno 2. The random assortment of the two enolase polypeptides into catalytically active dimers results in the presence of three enolase isozymes in the cell (Holland *et al.*, 1981; McAlister and Holland, 1982). Biochemical characteristics of these different yeast enolase isozymes such as pH optimum, Michaelis constant and inhibition by zinc were found to be identical (Pfleiderer *et al.*, 1968). Observations of the amino acid composition of the different yeast enolase isozymes showed the amino acid composition to be nearly identical (Westhead and McLain, 1964). The polypeptides predicted from the primary structures of the enolase genes differ at only 20 of the 436 amino acid residues present (Holland *et al.*, 1981). From these results, it appears that the isozymes of yeast enolase have no significance in metabolic regulation. However, the expression of the yeast enolase genes, *in vivo*, is highly dependent on the carbon source used to propagate the cells. The amounts of the enolase 1 polypeptide were similar in cells grown on glucose (fermentable carbon source) and in cells grown on either ethanol or glycerol plus lactate (non-fermentable carbon source). In contrast, the amounts of the enolase 2 polypeptide was twenty times higher in cells grown on glucose than in cells grown on ethanol or glycerol plus lactate. The steady-state concentrations of both the enolase 1 polypeptide and enolase 2 polypeptide were similar in cells grown on the non-fermentable carbon sources.

Total cellular RNA was isolated from cells grown on glucose and ethanol respectively and subjected to *in vitro* translation. The distributions of the

polypeptides synthesized *in vitro* and *in vivo* were similar. This indicates that the distribution of the two enolase mRNAs in the cells grown on different carbon sources is the same as the distribution of the two polypeptides in the cell (McAlister and Holland, 1982). Therefore, it seems that the transcription of the two enolase genes in glycolytic carbon sources and gluconeogenic carbon sources differs from one another.

Most mutations affect single enzymes, but *gcr* ("glycolysis regulation") is a mutation which seems to decrease the level of most enzymes in the glycolytic pathway (Clifton *et al.*, 1978). Studies carried out on the *gcr-1* gene (Holland *et al.*, 1987) show that the gene encodes a trans-acting factor which is required for the expression of enolase and glyceraldehyde-3-phosphate dehydrogenase. Yeast strains carrying the *gcr-1* mutation showed a marked decrease in the intracellular concentrations of the two enolases (Eno 1 and Eno 2) and the three glyceraldehyde-3-phosphate dehydrogenase genes (TDH1, TDH2 and TDH3). The expression of the enolase genes has been shown to be positively regulated by the presence of cis-acting sequences present within the 5' flanking regions of these genes (refer Holland *et al.*, 1987; Cohen *et al.*, 1986). Hence, there is a possibility that the product of the *gcr-1* gene regulates, to a certain extent, the activity of the cis-acting regulatory sequences. On the other hand, it is possible that the *gcr-1* gene product may regulate, also to a certain extent, the binding of another protein to the cis-acting regulatory sequences. Although, further studies are required to confirm any of the above possibilities, the *gcr-1* gene product has an important role to play in coordinating the regulation of transcription of many yeast glycolytic genes (Holland *et al.*, 1987).

Mammalian tissues such as rat brain and liver contain three types of isozymes composed of homodimers i.e. $\alpha\alpha$, $\beta\beta$ and $\gamma\gamma$ (Kato *et al.*, 1983). The $\alpha\alpha$ isozyme (non-neuronal enolase) is found in various tissues within the adult nervous tissues. The $\beta\beta$ isozyme is localized in muscle tissue and the $\gamma\gamma$ isozyme (neurone-specific enolase) is found only in neurons and neuroendocrine cells in the nervous tissue.

In *E. coli*, only one form of the enolase has been found (Pfleiderer *et al.*, 1966; Spring and Wold, 1971). These results are consistent with the studies carried out by Irani and Maitra (1974, 1976) which indicate the presence of a single enolase locus in *E. coli*.

A comparison of the structure of the *E. coli* enolase gene with its eukaryotic counterparts may provide some insights into the phylogenetic origins of prokaryotes and eukaryotes. In addition, it may also yield valuable information concerning the differences in regulation and expression of glycolytic genes in prokaryotes and eukaryotes. As a start to comparing the structure of the *E. coli* enolase gene with its eukaryotic counterparts, the gene was successfully isolated as part of the plasmid pST 34. When extracts of *E. coli* containing pST 34 or other hybrid plasmids were assayed for enolase activity, that of pST 34 showed an increase in enzyme activity over and above those of the other transformants. The plasmid pST 34 was digested with the restriction endonuclease, Eco RI to yield two fragments, E1 and E2. E1 was self-ligated and E2 was ligated to pUC 12 which had previously been cut with Eco RI. Frozen competent *E. coli* DH1 cells were then transformed with the ligated species to give transformants pST 34-E1

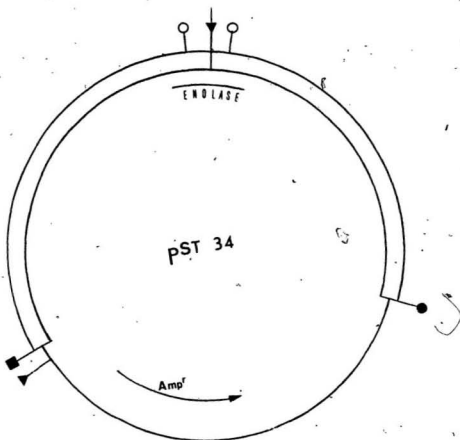
and pST 34-E2. Cell extracts of both pST 34-E1 and pST 34-E2, when assayed, did not show any increase in enolase activity. This therefore implies that the enolase gene or some regulatory region of the gene had been split when the plasmid pST 34 was digested with EcoRI (Figure 4-21).

Figure 4-21:

Enolase gene contained within pST 34.

The enolase gene is inactivated by digestion with Eco RI.

The symbols for the restriction enzyme sites are as follows: Bam
HI (H), Eco RI (R), Hind III (H) and Pst I (P).



Chapter 5

Characterization of a Multimeric Enolase from *S. acidocaldarius*

5.1. Background

A group of extreme thermoacidophilic archaeobacteria comprising the genus *Sulfolobus*, has been isolated from solfataric hot springs in Yellowstone National Park by Brock *et al.* (1972). This new genus of sulphur-oxidizing bacteria, is generally found in solfatara areas and can be readily isolated from a wide range of naturally acidic thermal habitats, both aquatic and terrestrial. Nearly all of the springs in which *Sulfolobus* was found were of low pH (less than 3.0) and high temperature (65 - 90°C). The pH optimum of *Sulfolobus* was found to be 2.0-3.0 and ranged from 0.9-5.8; the optimum temperature was 70-75°C and ranged from 55-85°C.

Members of the thermophilic eubacteria, of the genus *Thermus*, were first isolated from effluents of hot springs of neutral to alkaline pH (Brock and Freeze, 1969) and were later also isolated from man-made thermal habitats like hot water tanks and hot water heaters (Brock and Boyler, 1973). They have also been found in natural waters subject to thermal pollution. These bacteria have an optimum temperature of 70-75°C, and ranges from 40-70°C; the optimum pH for growth is 7.5-7.8 and ranges from 6.0-9.5.

An unusual feature of the enolases from *Thermus* is that they are octameric proteins with molecular weights of approximately 360,000 rather than dimers of about 90,000 as is the norm (Wold, 1971). It was of interest therefore, to compare the enolase from *Sulfolobus acidocaldarius*, a sulphur-dependent archaebacterium that lives in hot springs at temperatures up to 87°C and at a pH of approximately 2.0-2.5 (Zillig et al., 1980), with enolases that had been characterized from the extreme thermophiles, *Thermus aquaticus* YT-1 and *Thermus* X-1 (Stellwagen et al., 1973; Barnes and Stellwagen, 1973) to see if these enolases share any structural properties.

5.2. Approach of Investigation

To be able to compare properties of the enolase from *Sulfolobus acidocaldarius* with that of the enolases from the genus, *Thermus*, it was important to first purify the enzyme and then characterize it in terms of its native molecular weight, subunit structure and kinetic parameters such as pH optimum, effect of substrate concentration and the Michaelis constant for the substrate, 2-PGA. The requirement of magnesium ions for maximal activity of the enzyme and the thermal stability of the enolase from *Sulfolobus acidocaldarius* were also investigated.

5.3. Results

5.3.1. Purification

There was no discernable enolase activity observed in the dialysed post-ribosomal cytoplasmic extract from *S. acidocaldarius*. This may have been, due to the presence of other glycolytic enzymes, which utilize PEP as a substrate (i.e. rate of utilization being equal to that of its production), and hence make it difficult for the rate of enzyme activity to be measured. Or it may simply have been that the enzyme concentration was too low and the background very high which again would mask any enolase activity present. However, when the dialysed extract was applied to a DEAE-cellulose column and eluted step-wise with increasing concentrations of salt in Buffer A, fractions exhibiting enolase activity were obtained at a salt concentration of 0.25M NaCl (Figure 5-1).

These fractions were pooled, concentrated by ultrafiltration and further purified by gel filtration (Figure 5-2) and ion-exchange chromatography using a linear salt gradient to elute the enzyme (Figure 5-3). Fractions containing enzyme activity were pooled, concentrated and applied to a Sephacryl S-300 column.

Table 5-1 summarizes the purification procedure. Although the overall purification appears to be low, it is important to note that as the starting material was a post-ribosomal cytoplasmic extract, a considerable amount of protein had already been removed during the isolation of the ribosomes. In addition to this, one must remember that the reference material is the active fraction from the first DEAE-cellulose column and a lot of protein had been removed in this first step.

Figure 5-1:

Step-wise elution of dialysed extract from DEAE-cellulose (DE52) column.

The letters correspond to the following:

A: Buffer A

B: Buffer A containing 0.1 M NaCl

C: Buffer A containing 0.25 M NaCl

D: Buffer A containing 1.0 M NaCl

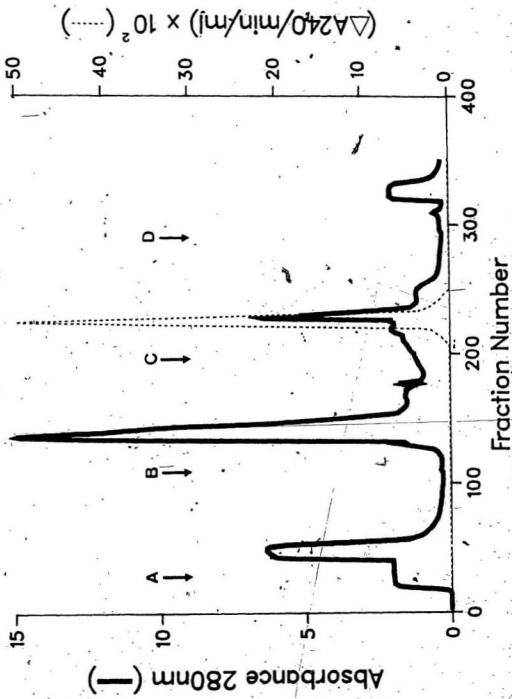


Figure 5-2: Purification of the *S. acidocaldarius* enolase by gel filtration on Sephacryl S-300.

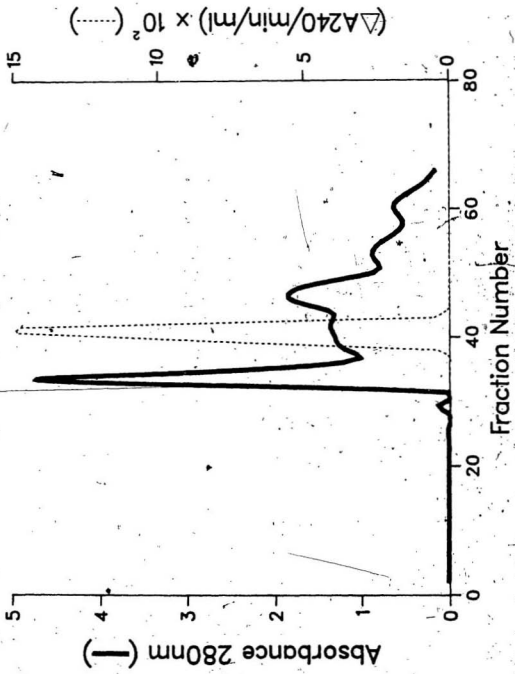


Figure 5-3:

Elution of the *S. acidocaldarius* enolase from DEAE-cellulose using a linear salt gradient.

A: Buffer A

B: Buffer A containing 0.1 M NaCl

C: Buffer A containing a linear gradient of 0.1 - 0.3 M NaCl

D: Buffer A containing 1.0 M NaCl

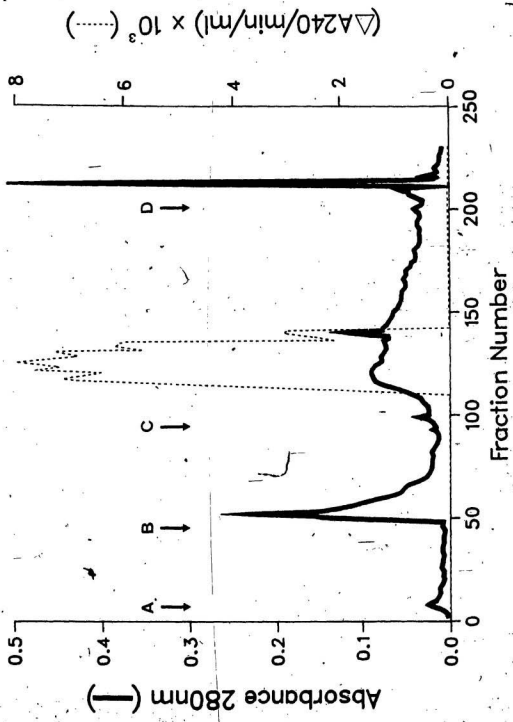


Table 5-1: Purification of enolase from *S. acidocaldarius*

Fraction	Total Activity $\mu\text{mol}\cdot\text{min}^{-1}$	Total Protein mg	Specific Activity $\mu\text{mol}\cdot\text{min}^{-1}/\text{mg}$	Fold Purification	Percent Recovery
DEAE-Cellulose	4.35	189.00	0.023	1	100
S-300	1.42	21.17	0.067	3	33
DEAE-Cellulose	0.97	4.2	0.231	10	22
S-300	1.07	0.95	1.126	50	25

To determine the subunit molecular weight of the enolase further purification of the enzyme, after ion-exchange chromatography using a linear salt gradient, was achieved as follows. The active fractions were pooled, concentrated and dialysed overnight against Buffer B at pH 6.5. The dialysed fraction was then applied to a phospho-cellulose column and fractions containing enzyme activity were eluted with Buffer B containing 1 M NaCl (Figure 5-4). The final purification step involved concentrating the pooled fractions containing enolase activity and applying the concentrated protein to a gel filtration column (Figure 5-5).

5.3.2. Extinction Coefficient of PEP

The extinction coefficient of PEP varies with pH and the concentration of magnesium ions present (Wold and Ballou, 1957). The extinction coefficient of PEP was determined at different pHs in the presence and absence of magnesium ions, but no dependence on the magnesium ion concentration was observed (Figure 5-6). Activity measurements were corrected accordingly using the value of the extinction coefficient at the appropriate pH. These measurements were expressed in terms of μ moles of PEP produced per minute.

5.3.3. Kinetic Parameters

Figure 5-4:

Profile of enolase activity from Phospho-cellulose column.

A: Buffer B containing 1.0 M NaCl.

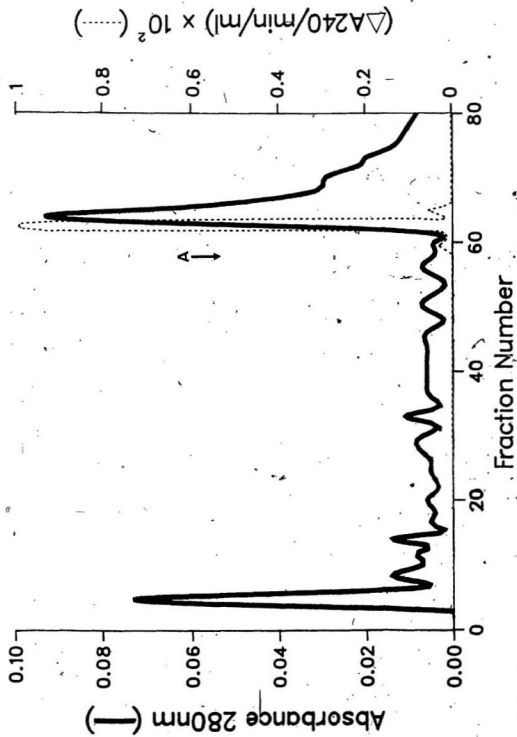
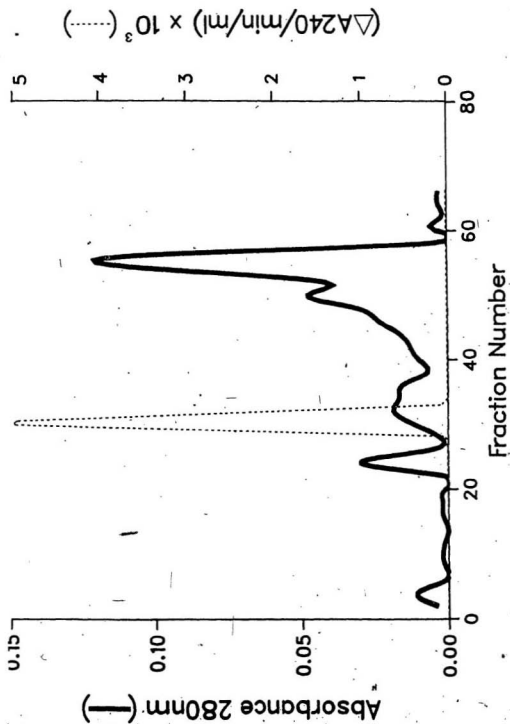


Figure 5-5. Profile of *S. acidocaldarius* enolase from
Sephacryl S-300



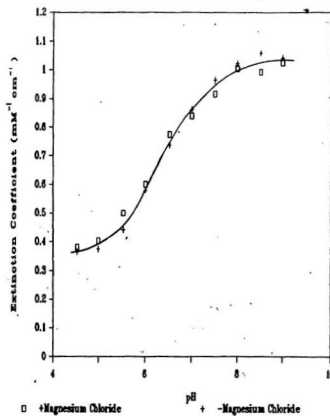


Figure 5-6: pH dependence of the extinction coefficient of PEP

5.3.3.1. Effect of pH

Enzyme assays were carried out in Tris/acetate buffers of varying pHs from 5.0 to 9.0. The pH optimum for the forward reaction catalysed by the enolase from *S. acidocaldarius* is 6.5 (Figure 5-7). This is lower than the pH optima for the enolases from *Thermus* species and *Escherichia coli* (Table 5-2)

5.3.3.2. Effect of Magnesium Ion Concentration

It had been previously shown that enolase required the presence of magnesium ions for maximal activity (Pietkiewicz *et al.*, 1983). To observe the effect of magnesium ion concentration on the activity of the enolase from *S. acidocaldarius*, enzyme assays were carried out at different concentrations of magnesium ions ranging from 0.5 - 20 mM. Figure 5-8 shows a plot of the results obtained. Increasing enzyme activity was observed with increasing magnesium ion concentration from 0.5 - 10 mM. The magnesium ion concentration required for maximal activity of the enzyme is 10 mM. In order to help stabilize the enolase during the purification, the buffers employed contained magnesium chloride at a concentration of 1.0 mM.

5.3.3.3. Effect of Substrate Concentration

The effect of different concentrations of the substrate, 2-PGA on the activity of the enzyme was also investigated. The substrate concentrations varied from 0.005 - 2.0mM. The results obtained (Figure 5-9) show that the enzyme obeyed Michaelis-Menten kinetics. Maximum activity was observed at substrate concentrations of 1.0 mM and 2.0 mM.

The Michaelis constant for 2-PGA for the enolase from *S. acidocaldarius*

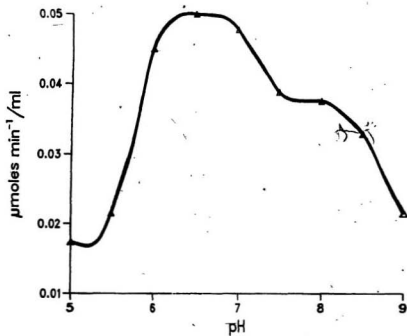


Figure 5-7: Effect of pH on the activity of the *S. acidocaldarius* enolase

Table 5-2: Properties of enolases from different organisms

Organism	pH optimum	K_m DPGA (mM)	Molecular Weight		Reference
			Native	Subunit	
Yeast	7.7	0.19	88,000	44,000	Pietkiewicz <i>et al.</i> , 1983
<i>E. coli</i>	8.1	0.10	90,000	46,000	Pietkiewicz <i>et al.</i> , 1983
<i>T. X-1</i>	8.3	0.15	384,000	48,000	Barnes and Stellwagen, 1973
<i>T. aquaticus</i>	8.3	0.10	352,000	44,000	Stellwagen <i>et al.</i> , 1973
<i>S. acidocaldarius</i>	6.5	0.02	400,000	45,700	Present study

Effect of Magnesium ion concentration

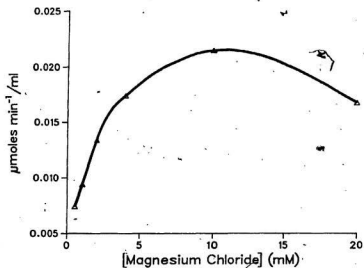


Figure 5-8: Effect of magnesium ion concentration on the activity of enolase from *S. acidocaldarius*

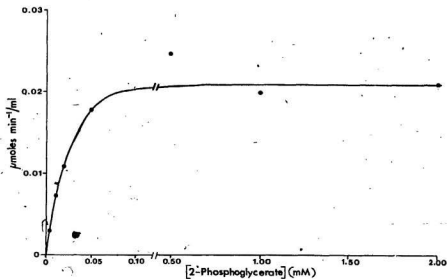


Figure 5-9: Effect of substrate concentration on the activity of the enolase from *S. acidocaldarius*

was determined using a nonlinear regression curve fitting package [ROSFIT] which has been specifically designed for enzyme kinetic analysis with a Hewlett-Packard HP 85 microcomputer (Greco *et al.*, 1982). The K_m obtained was 0.021 ± 0.004 mM, which is not significantly different from those of other bacteria (see Table 5-2).

5.3.4. Physical Characteristics

5.3.4.1. Native Molecular Weight

Gel filtration chromatography on Sephacryl S-300 revealed that the enolase from *S. acidocaldarius* has an apparent native molecular weight of 400,000 (Figure 5-10). This result is similar to the molecular weight of the enolases from *Thermus* which are approximately 360,000. In contrast, when rabbit enolase was applied to the same Sephacryl S-300 column it yielded an apparent molecular weight of 82,000.

5.3.4.2. Subunit Structure

When the enolase from *S. acidocaldarius* was subjected to SDS PAGE, the results showed two minor protein bands and a major protein band. The two minor bands are probably contaminants since the enzyme was not completely purified. The major protein band migrated approximately the same distance as the enolase from yeast (Figure 5-11). The apparent molecular weight of this protein is approximately 45,700 (Figure 5-12). The enolase from the thermophilic eubacterium, *Thermus aquaticus*, consists of eight polypeptide chains each with a molecular weight of 44,000 (Stellwagen *et al.*, 1973). The results indicate that the major band seen on the gel in Figure 5-10 is probably enolase. This suggests that

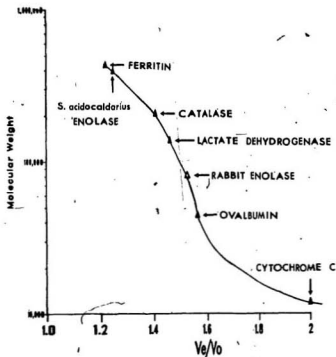


Figure 5-10: Determination of the native molecular weight of the enolase from *S. acidocaldarius*

the enolase isolated from the archaeobacterial thermoacidophile, *S. acidocaldarius*, is a multimeric enzyme and is likely to be an octamer as are its eubacterial thermophilic counterparts.

5.3.5. Heat Denaturation Studies

The thermal stability of the enolase from *S. acidocaldarius* was compared with that of the enolase from rabbit muscle. Samples of the enzyme were heated at varying temperatures for five minutes and cooled in an ice-water bath for fifteen minutes. Samples were then assayed at 25°C for enolase activity. Figure 5-13 shows the temperature inactivation profiles of the enolases from *S. acidocaldarius* and rabbit muscle. The temperature at which fifty percent of the activity has been lost in five minutes, ($A_{1/2}$), is 88°C for the enolase from *S. acidocaldarius* compared to 75°C for the rabbit muscle enolase. Note too that no activity was lost at temperatures up to 80°C for the *S. acidocaldarius* enolase. Hence, the enzyme from *S. acidocaldarius* was found to be more stable at higher temperatures than the enolase from rabbit muscle. These results are similar to those obtained by Stellwagen *et al.* (1973) and Barnes and Stellwagen (1973) with the enolases from *Thermus* species.

The mechanisms whereby proteins achieve increased thermal stability are still unclear. However, Barnes and Stellwagen (1973) noted that in enolases there is a negative correlation between $A_{1/2}$ and the average hydrophobicity and a positive correlation between $A_{1/2}$ and the sum of the amino acid residues capable of forming hydrogen bonds. It was not possible to test either of these observations as the enolase from *S. acidocaldarius* was not purified to homogeneity.

Figure 5-11:

SDS-PAGE analysis of the subunit molecular weight of the *S. acidocaldarius* enolase.

The molecular weight markers used were:

Lane 1: Ovalbumin dimer (86,000), ovalbumin (43,000), chymotrypsinogen A (25,700), β -lactoglobulin (18,400) and lysozyme (14,300)

Lane 2: Yeast enolase (47,000) from Sigma

Lane 3: 25 μ g of freeze dried *S. acidocaldarius* enolase

Lane 4: 50 μ g of freeze dried *S. acidocaldarius* enolase

1 2 3 4

86,000 →

47,000 →

43,000 →

25,700 →

18,400 →

14,300 →

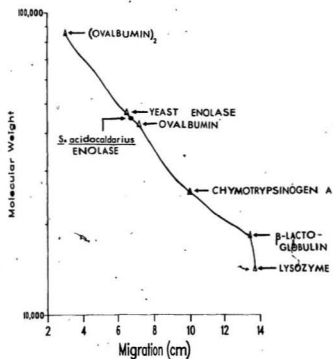


Figure 5-12: Determination of the subunit molecular weight of the *S. acidocaldarius* enolase

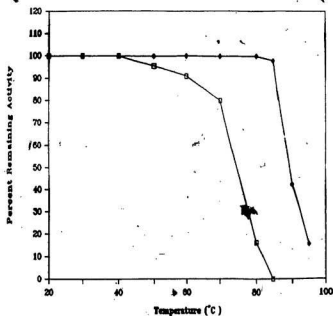


Figure 5-13: Effect of temperature on the stability of the enolases from rabbit muscle (□) and *S. acidocaldarius* (●)

5.4. Discussion

The optimum pH of the enolase from *S. acidocaldarius* is an acidic pH of 6.5. The pH optima for the other organisms were alkaline. For example, the pH optimum for the enolase from *E. coli* is 8.1 and that for the *Thermus* species is 8.3. Since the genus *Sulfolobus* is found in acidic habitats, this low pH may reflect an adaptation of the organism to the acidic environment in which it lives.

The magnesium ion concentration required for maximal activity of the enzyme is 10 mM. Magnesium ions are not only important catalytically but also structurally as they stabilize the interactions of the subunits in the enzyme. The substrate concentration at which maximal activity of the enzyme was observed was between 1.0 and 2.0 mM. Based on the data obtained for the activity of the enzyme at different substrate concentrations, the Michaelis constant was calculated to be 0.026 mM. In both *E. coli* and *T. aquaticus* the K_m for 2-PGA is 0.1 mM. The K_m of 2-PGA obtained with the *S. acidocaldarius* enolase was not significantly different when compared to the K_m from the other organisms.

Studies carried out on the thermal stability of the enolase from *S. acidocaldarius* showed that the enzyme was active at temperatures up to 80°C without any loss of activity whatsoever. The temperature at which fifty per cent of the activity had been lost in five minutes ($A_{1/2}$) was 88°C. Thereafter, pre-incubations at higher temperatures resulted in a progressive loss of activity. Rabbit muscle enolase was used as a control and it showed an $A_{1/2}$ at 75°C. Similar results were obtained by Stellwagen *et al.* (1973) and Barnes and Stellwagen (1973) on studies carried out with the enolase from *T. aquaticus*. The

initial activity of the enzyme was completely retained after incubations at 75°C and 85°C. The $A_{1/2}$ of the *T. aquaticus* enolase was 90°C. The results demonstrate the thermostability of the *S. acidocaldarius* enolase and reflect the temperature at which *Sulfolobus* naturally thrives.

The apparent native molecular weight of the *S. acidocaldarius* enolase is 400,000, which is comparable to the native molecular weight of 360,000 obtained from the *T. aquaticus* enolase. In contrast, the native molecular weight of enolases from other organisms range from 80,000 - 100,000. SDS PAGE of the enolase from *S. acidocaldarius* showed that the major protein band migrated approximately the same distance as the enolase from yeast. The approximate molecular weight of this protein is 45,700. Therefore, the results indicate that like the enolases from eubacterial thermophiles, the enolase from *S. acidocaldarius*, a thermophilic archaeobacterium, is a multimeric enzyme, most probably an octamer.

As a matter of interest, the results obtained with the enolase from *S. acidocaldarius*, a thermophilic archaeobacterium, are similar to those obtained with the enolases from *T. aquaticus*, a eubacterial thermophile. This suggests therefore, that the enolases that are multimeric in nature are thermally more stable. Whether or not this is a common mechanism in adapting to life at high temperatures remains to be seen, but it is certainly noteworthy that the enolases from such phylogenetically divergent thermophiles should share this multimeric feature.

Macromolecules of thermophiles are stable at the high temperatures at which the organisms live. However, thermophily is not confined to a single phylogenetical line of bacteria. It is found in both archaeobacteria and eubacteria (Brock, 1985). From this one can postulate that perhaps a common ancestral organism of both the archaeobacteria and eubacteria was a thermophile. The question then arises of whether or not *Sulfolobus* is really an archaeobacterium. However, one cannot speculate further on this without additional evidence.

Chapter 6

Conclusions and Future Directions

The *E. coli* enolase gene was successfully isolated as part of the plasmid pST 34. The results obtained with the hybrid plasmids pST 34-E1 and pST 34-E2 indicated that the enolase gene or some regulatory region of the gene had been split when the plasmid pST 34 was digested with Eco RI. Therefore, having successfully located the enolase gene to the region around the unique Eco RI site of the insert contained in pST 34, the next step in this project would be to sequence the gene. The best way to go about it would be to separately sequence the inserts contained in pST 34-E1 and pST 34-E2-A and combine the sequences obtained to get the DNA sequences of the enolase gene. This can be done in two ways.

In the first method, the hybrid plasmid pST 34-E1 is cleaved with Eco RI to give a linear fragment. The 5' phosphates are then removed by treating the fragment with calf intestinal phosphatase. The 5' ends of the fragment can then be labelled by incubating the DNA with radioactively labelled [γ - 32 P]-ATP and T_4 polynucleotide kinase. The labelled fragment is then cleaved with Hind III to yield two fragments, each labelled at their Eco RI 5' ends. The fragments can be individually isolated and each subjected to the chemical DNA sequencing method

(Maxam and Gilbert, 1980). This will give the DNA sequence of part of the enolase gene contained in pST 34-E1.

In the second method, the plasmid pST 34-E2-A can be subjected to the enzymatic sequencing technique (Sanger and Coulson, 1977). This would involve the use of a primer, dideoxynucleotides and DNA polymerase I (Klenow). The sequences obtained from pST 34-E1 and pST 34-E2-A can then be joined together and compared with the DNA sequences of the enolase genes from other organisms. The sequencing of the enolase gene from pST 34 is presently being done in Dr. W. S. Davidson's laboratory.

The enolase from *S. acidocaldarius*, a thermophilic archaebacterium has been characterized with respect to its native and subunit molecular weight. Kinetic parameters such as the K_m for 2-PGA, pH optimum, magnesium dependence and thermal stability of the enzyme were determined and compared with those of the enolases from eubacteria and eukaryotes. The results obtained showed that the enolases from *S. acidocaldarius* and members of the eubacterial genus *Thermus* are unusual in that they are thermostable and exist as octamers whereas the enolases from other organisms exist as dimers. Further work in this study would involve characterizing the enolases from other thermophilic archaebacteria and eubacteria to see whether or not this is a common trend among bacteria that live at high temperatures. If the enolases from *S. acidocaldarius* and other thermophilic bacteria were isolated in a pure form, it would also be possible to test the hypothesis of Stellwagen *et al.* (1973) that there are correlations between the thermostabilities of proteins and their average hydrophobicities and their potential for hydrogen bond formation.

Chapter 7

References

1. Barnes, L.D. and Stellwagen, E. 1973. Enolase from the thermophile *Thermus X-1*. Biochemistry 12: 1559-1565.
2. Birnboim, H.C. and Doly, J. 1979. A rapid alkaline extraction procedure for screening recombinant DNA. Nucleic Acids Research 7: 1513-1523.
3. Boser, H. 1959. The heterogeneity of enzymes. I. Enolase of potato tubers. Z. Physiol. Chem. 315: 163-170.
4. Brock, T.D. 1985. Life at high temperatures. Science 230: 132-138.
5. Brock, T.D. and Freeze, H. 1969. *Thermus aquaticus* gen. n. and sp. n., a nonsporulating extreme thermophile. J. Bact. 98: 289-297.
6. Brock, T.D. and Boylen, K.L. 1973. Presence of thermophilic bacteria in laundry and domestic hot-water heaters. App. Microb. 25: 72-76.
7. Brock, T.D., Brock, K.M., Belley, R.T. and Weiss, R.L. 1972. *Sulfolobus*: A new genus of sulfur-oxidizing bacteria living at low pH and high temperature. Arch. Mikrobiol. 84: 54-68.
8. Brewer, J.M. and Weber, G. 1968. The reversible dissociation of yeast enolase. Proc. Natl. Acad. Sci. U.S.A. 59: 216-223.
9. Chin, C.C., Brewer, J.M., Eckard, E. and Wold, F. 1981a. The amino acid sequence of yeast enolase: Preparation and characterization of peptides produced by chemical and enzymatic fragmentations. J. Biol. Chem. 256: 1370-1376.
10. Clarke, L. and Carbon, J. 1976. A colony bank containing synthetic Col E1 hybrid plasmid representative of the entire *E. coli* genome. Cell 9: 91-99.

11. Clifton, D., Weinstock, S.B. and Fraenkel, D.G. 1978. Glycoysis mutants in *Saccharomyces cerevisiae*. *Genetics* 88:1-11.
12. Cohen, R., Holland, J.P., Yokoi, T. and Holland, M.J. 1988. Identification of a regulatory region that mediates glucose-dependent induction of the *Saccharomyces cerevisiae* enolase gene *ENO 2*. *Mol. Cell. Biol.* 6: 2287-2297.
13. Cory, R.P. and Wold, F. 1966. Isolation and characterization of enolase from rainbow trout. [*Salmo gairdnerii gairdnerii*]. *Biochemistry* 5: 3131-3137.
14. Dickerson, R.E. and Geis, I. 1969. *The Structure and Action of Proteins*, W.A. Benjamin, Inc., Menlo Park, California.
15. Fraenkel, D.G. and Vinopal, R.T. 1973. Carbohydrate metabolism in bacteria. *Ann. Rev. Microbiol.* 27: 69-100.
16. Fox, G.E., Stackebrandt, E., Hespell, R.B., Gibson, J., Maniloff, J., Dyer, T.A., Wolfe, R.S., Balch, W.E., Tanner, R.S., Magrum, L.J., Zablen, L.B., Blakemore, R., Gupta, R., Bonen, L., Lewis, B.J., Stahl, D.A., Leuhrsen, K.R., Chen, K.N. and Woese, C.R. 1980. The phylogeny of prokaryotes. *Science* 209: 457-463.
17. Futuyma, D.J. 1983. *Science on Trial*, p.73., Pantheon Books, New York.
18. Gawronski, T.H. and Westhead, E.W. 1969. Equilibrium and kinetic studies on the reversible dissociation of yeast enolase by neutral salts. *Biochemistry* 8: 4261-4270.
19. Greco, W.R., Priore, R.L., Sharma, M. and Korytnyk, W. 1982. ROSFIT: an enzyme kinetics nonlinear regression curve fitting package for a microcomputer. *Comput. Biomed. Res.* 15: 39-45.
20. Gupta, R., Lanter, J.M. and Woese, C.R. 1983. Sequence of the 16S ribosomal RNA from *Halobacterium volcanii*, an Archaeobacterium. *Science* 221: 656-659.
21. Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* 166: 557-580.
22. Hanlon, D.P. and Westhead, E.W. 1969. Equilibrium measurements of the interaction of yeast enolase with activating metal ions. *Biochemistry* 8: 4247-4255.

23. Holland, M.J., Holland, J.P., Thill, G.P. and Jackson, K.A. 1981. The primary structures of two yeast enolase genes. *J. Biol. Chem.* 256: 1385-1395.
24. Holland, M.J., Yokoi, T., Holland, J.P., Myambo, K. and Innis, M.A. 1987. The *GCR1* gene encodes a positive transcriptional regulator of the enolase and glyceraldehyde-3-phosphate dehydrogenase gene families in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 7: 813-820.
25. Irani, M.H. and Maitra, P.K. 1974. Isolation and characterization of *Escherichia coli* mutants defective in enzymes of glycolysis. *Biochem. Biophys. Res. Commun.* 56: 127-133.
26. Irani, M.H. and Maitra, P.K. 1976. Glyceraldehyde 3-P Dehydrogenase, glyceralate 3-P kinase and enolase mutants of *Escherichia coli*: Genetic studies. *Molec. Gen. Genet.* 145: 65-71.
27. Kato, K., Okagawa, Y., Suzuki, F., Shimizu, A., Mokuno, K. and Takahashi, Y. 1983. Immunoassay of human-muscle enolase subunit in serum - a novel marker antigen for muscle diseases. *Clin. Chim. Acta.* 131: 75-85.
28. Kimura, M. and Langner, G. 1984. The complete amino acid sequence of the ribosomal protein HS3 from *Halobacterium marismortui*, an archaeobacterium. *FEBS Letters* 175: 213-218.
29. Kun, E. 1950. Conversion of 3-phosphoglycerate to phosphoenolpyruvate by tissue homogenates. *Proc. Soc. Exptl. Biol. Med.* 75: 68-71.
30. Lake, J. A., Clark, M.W., Henderson, E., Fay, S.P., Oakes, M., Scheinman, A., Thornber, J.P. and Mah, R.A. 1985. Eubacteria, halobacteria, and the origin of photosynthesis: The photocytes. *Proc. Natl. Acad. Sci. USA* 82: 3716-3720.
31. Lake, J.A. 1986. An alternative to archaeobacterial dogma. *Nature* 319: 626.
32. Lake, J.A. 1986. In defence of bacterial phylogeny. *Nature* 321: 657-658.
33. Laemmli, U.K. 1970. Cleavage of structural proteins during assembly of bacterial phage T₄. *Nature* 227: 680-685.

34. Lederer, H. 1986. Archaeobacterial status quo is defended. *Nature* 320: 220.
35. Lohmann, K. and Meyerhof, O. 1934. Enzymic transformation of phosphoglyceric acid into pyruvic and phosphoric acids. *Biochem. Z.* 273: 60-72.
36. Looi-Lyons, L. 1987. M.Sc Thesis.
37. Malmstrom, B.G. 1957. The purification of yeast enolase by zone electrophoresis and ion-exchange chromatography, and the existence of several active forms of the enzyme. *Arch. Biochem. Biophys.* 70: 58-69.
38. Maniatis, T., Fritsch, E.F. and Sambrook, J. 1982. In *Molecular Cloning Laboratory Manual*. Cold Spring Harbor Laboratory Publication.
39. Maxam, A.M. and Gilbert, W. 1980. Sequencing end-labelled DNA with base specific chemical cleavages. *Methods Enzymol.* 65: 499-560.
40. McAlister, L. and Holland, M.J. 1982. Targeted deletion of a yeast enolase structural gene. *J. Biol. Chem.* 257: 7181-7188.
41. Miernyk, J.A. and Dennis, D.T. 1984. Enolase isozymes from *Ricinus communis*: Partial purification and characterization of the isozymes. *Arch. Biochem. Biophys.* 233: 643-651.
42. Pfeleiderer, G., Neufahrt-Kreiling, A., Kaplan, R.W. and Fortnagel, P. 1966. Biochemical, immunological, and genetic investigations of the multiple forms of yeast enolase. *Biochem. Z.* 346: 269-281.
43. Pfeleiderer, G., Neufahrt-Kreiling, A., Kaplan, R.W. and Fortnagel, P. 1968. Biochemical immunological and genetic investigations of the multiple forms of yeast enolase. *Ann. N.Y. Acad. Sci.* 151:78-84.
44. Pietkiewicz, J., Kustrzeba-Wojcicka, I. and Wolna E. 1983. Purification and properties of enolase from carp *Cyprinus carpio* comparison with enolases from mammals' muscles and yeast. *Comp. Biochem. Physiol.* 75B: 693-698.
45. Ruth, R.C., Soja, D.M. and Wold, F. 1970. Purification and characterization of enolase from Coho *Oncorhynchus kisutch* and Chum *Oncorhynchus keta* salmon. *Arch. Biochem. Biophys.* 140: 1-10.

46. Sakimura, K., Kushiya, E., Obinata, M. and Takahashi, Y. 1985. Molecular cloning and the nucleotide sequence of cDNA to mRNA for non-neuronal enolase ($\alpha\alpha$ enolase) of rat brain and liver. *Nucleic Acids Research* 13: 4365-4378.
47. Sanger, N. and Coulson, A.R. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci.* 74: 5463-5467.
48. Spring, T. G. and Wold, F. 1971. The purification and characterization of *Escherichia coli* enolase. *J. Biol. Chem.* 246: 6797-6802.
49. Stellwagen, E., Cronlund, M.M. and Barnes, L.D. 1973. A thermostable enolase from the extreme thermophile *Thermus aquaticus* YT-1. *Biochemistry* 12: 1552-1559.
50. Thomson, J., Gerstenberfer, P.D., Goldberg, D.E., Gociar, E., de Silva, A.O. and Fraenkel, D.G. 1979. Col E1 hybrid plasmids for *Escherichia coli* genes of glycolysis and the hexose monophosphate shunt. *J. Bacteriol.* 137: 502-506.
51. Tsuyuki, H. and Wold, F. 1964. Enolase: Multiple molecular forms in fish muscle. *Science* 146: 535-537.
52. Van Valen, L.M. and Maiorana, V.C. 1980. The archaeobacteria and eukaryotic origins. *Nature* 287: 248-250.
53. Westhead, E.W. and McLain, G. 1964. A purification of Brewers' and Bakers' yeast enolase yielding a single active component. *J. Biol. Chem.* 239: 2464-2468.
54. Wilson, A.C., Carlson, S.S., and White, T.J. 1977. Biochemical evolution. *Ann. Rev. Biochem.* 46: 573-639.
55. Winstead, J.A. and Wold, F. 1965. Dissociation and reassociation of rabbit muscle enolase. *Biochemistry* 4: 2145-2158.
56. Woese, C.R. and Fox, G.E. 1977. Phylogenetic structure of the prokaryotic domain: The primary kingdoms. *Proc. Natl. Acad. Sci. U.S.A.* 74: 5088-5090.
57. Woese, C.R., Pace, N.R. and Olsen, G.J. 1986. Are arguments against archaeobacteria valid? *Nature* 320: 401-402.

58. Wold, F. 1971. Enolase. In *The Enzymes* (Edited by Boyer, P.D.) 3rd edition, vol 5, pp499-538. Academic Press, New York.
59. Wold, F. and Ballou, C.E. 1957a. Studies on the enzyme enolase. *J. Biol Chem.* 227: 301-312.
60. Wold, F. and Ballou, C.E. 1957b. Studies on the enzyme enolase. *J. Biol. Chem.* 227: 313-322.
61. Wold, F. and Ballou, C.E. 1959. The DL-2,3-Dihydroxyisobutyric acid monophosphates. *J. Am. Chem. Soc.* 81: 2368-2371.
62. Wood, T. 1964. The purification of enolase from cerebral tissue. *Biochem. J.* 91: 453-460.
63. Yaguchi, M., Visentin, P., Zuker, M., Matheson, A.T., Roy, C. and Strom, A.R. 1982. Amino-terminal sequences of ribosomal proteins from the 30S subunit of archaebacterium *Halobacterium cutirubrum*. *Zbl. Bakt. Hyg. I. Abt. Orig. C3*: 200-208.
64. Zillig, W. 1986. Archaeobacterial status quo is defended. *Nature* 320: 220.
65. Zillig, W., Stetter, K.O., Wunderl, S., Schultz, W., Priess, H. and Scholtz, I. 1980. The *Sulfolobus-Caldariella* group: Taxonomy on the basis of the structure of DNA-dependent RNA polymerases. *Arch. Microbiol.* 125: 259-269.

Chapter 8

Appendix I

1. LB(Luria-Broth) Medium

Bacto-tryptone
Bacto-yeast extract
NaCl

Per Litre:

10.0 g
5.0 g
10.0 g

Adjust to pH 7.2 with sodium hydroxide.

Autoclave at 15 lb/sq. in. for twenty minutes.

2. LB/Ampicillin Plates

Bacto-tryptone
Bacto-yeast extract
NaCl
Bacto-agar

Per Litre:

10.0 g
5.0 g
10.0 g
15.0 g

Adjust to pH 7.2 with sodium hydroxide.

Autoclave at 15 lb/sq. in. for twenty minutes.

Cool.

Add 10 ml ampicillin (2 mg/ ml).

Pour plates. Flame.

Let set.

3. ψb Media

Per Litre:

Bacto-tryptone	20.0 g
Bacto-yeast extract	5.0 g
MgSO ₄	5.0 g

Adjust to pH 7.6 with potassium hydroxide.

Autoclave at 15 lb/sq. in. for twenty minutes.

4. Tfb I

Per Litre:

KAc	30.0 mM
RbCl	100.0 mM
CaCl ₂ ·2H ₂ O	10.0 mM
MnCl ₂ ·4H ₂ O	50.0 mM
Glycerol	15%

Adjust to pH 5.8 with acetic acid (less than one drop)

Filter sterilize. Do not autoclave.

5. Tfb II

Per Litre:

Morpholinopropanesulfonic acid	10.0 mM
CaCl ₂ ·2H ₂ O	75.0 mM
RbCl	10.0 mM
Glycerol	15%

Adjust to pH 6.5 with fresh potassium hydroxide.

Filter sterilize.

6. 10X TBE (Tris/Borate)

Per Litre:

Trizma base	108.0 g
Boric acid	55.0 g
0.5 M EDTA (pH 8.0)	20.0 ml

7. 10X TAE (Tris/Acetate)

Per Litre:

Trizma base	48.4 g
Glacial acetic acid	11.4 ml
0.5 M EDTA (pH 8.0)	20.0 ml

8. TE (Tris/EDTA)

10.0 mM Tris/HCl (pH 8.0)

1.0 mM EDTA (pH 8.0)

9. Reservoir Buffer for SDS PAGE

Per Litre:

Glycine	28.8 g
Trizma base	6.0 g
0.25 M EDTA	8.0 ml
20% SDS	5.0 ml

10. 10X Ligation Buffer

0.5 M Tris/HCl (pH 7.5)

0.1 M Magnesium chloride

0.1 M Dithiothreitol

0.01 M Adenosine tri phosphate

